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AWARD NUMBER DAMD17-94-J-4159

TITLE: Role of Epidermal Growth Factor Receptors and Their Ligands in Normal Mammary Epithelial and Breast Cancer Cells

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REPORT DATE: July 1997

TYPE OF REPORT: Final

PREPARED FOR: Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

19971230 045

DTIC QUALITY INSPECTED 5

REPORT DOCUMENTATION PAGE

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE July 1997	3. REPORT TYPE AND DATES COVERED Final (1 Jul 94 - 30 Jun 97)

4. TITLE AND SUBTITLE
Role of Epidermal Growth Factor Receptors and Their Ligands in Normal Mammary Epithelial and Breast Cancer Cells

5. FUNDING NUMBERS
DAMD17-94-J-4159

6. AUTHOR(S)
Kathleen M. Darcy, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Health Research, Inc.
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING/MONITORING AGENCY NAME(S) AND ADDRESS(ES)
Commander
U.S. Army Medical Research and Materiel Command
Fort Detrick, Frederick, Maryland 2170-25012

10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, July 1997). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200
Epidermal growth factor (EGF) and transforming growth factor α (TGFα) acting through EGF receptors (EGFR) regulate the development of normal mammary epithelial cells (MEC) and breast cancer. Primary culture studies examined the effects of EGF and TGFα in MEC. PD158780, a selective inhibitor of the tyrosine kinase domain of EGFR, helped demonstrate that EGFR signaling was required for the proliferation and functional differentiation of immature MEC from days 0-7 of culture, and survival of terminally differentiated MEC from days 17.5-21 of the study. EGFR levels were expressed at high levels in non-functional MEC and MEC undergoing apoptosis whereas functionally differentiated MEC expressed relatively low levels of EGFR. In EGF medium, cultured MEC expressed peak levels of erbB2 and erbB3 from day 7-14 of culture, whereas erbB4 levels were only detected within MEC cultured for 7 and 10.5 days. MEC isolated from mammary glands expressed high levels of erbB2 and erbB3 in virgin rats and rats during pregnancy and involution, and relatively low levels during lactation, whereas erbB4 appeared to be uniformly expressed throughout the developmental stages analyzed. These findings will help in the development of therapies to treat aggressive human breast cancers that often overexpress EGFR and/or other erbB receptors.

14. SUBJECT TERMS Breast Cancer			15. NUMBER OF PAGES 94
			16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT Unclassified	18. SECURITY CLASSIFICATION OF THIS PAGE Unclassified	19. SECURITY CLASSIFICATION OF ABSTRACT Unclassified	20. LIMITATION OF ABSTRACT Limited

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INTRODUCTION

A. NATURE OF THE PROBLEM

For women in the United States, breast cancer incidence and mortality rates are unsettling. Incidence rates are among the highest in the world and continue to rise with time, while mortality rates remain unchanged. It is essential to identify the factors that stimulate or inhibit breast cancer progression, and to clarify the present understanding of the factors that regulate the proliferation, differentiation, adhesion, invasion and death of breast cancer cells and the normal cells from which this cancer is derived. Breast cancer development involves a progressive deregulation of the developmental pathways operative in normal mammary epithelial cells (MEC). Steroids, polypeptides and extracellular matrix (ECM) components coordinately regulate the development of the normal MEC as well as many breast cancer cells. Unfortunately, the mechanisms of action of these regulators, and their interactions are only partially understood. Breast cancer progression is classically characterized by a loss in responsiveness to ovarian steroids, growth inhibitors, and/or inducers of apoptosis, an upregulation in the expression of autocrine and paracrine growth factors, growth factor receptors and matrix remodeling enzymes, as well as an alteration in the adhesive properties of cells to other cells and to the different ECM components.

Epidermal growth factor (EGF) and transforming growth factor α (TGFα) have been shown to stimulate the development and malignant progression of breast cancer. These growth factors act by binding to the EGF receptor (EGFR), and activating its tyrosine kinase domain. A number of proteins involved in signal transduction have been shown to be tyrosine phosphorylated in response to EGFR activation by EGF. The functions and intracellular mechanisms of action of EGF and TGFα in normal MEC and breast cancer cells are not fully defined. Changes in the expression of EGFR and its ligands appear to be important in breast cancer progression. EGFR transcripts are overexpressed in ~50-60% of primary human breast tumors, and these carcinomas usually have an estrogen receptor and progesterone receptor negative phenotype, high proliferation rates, poor response to endocrine therapy, and reduced patient survival rates (1). Overexpression of EGFR confers a conditional ligand-dependent growth advantage to the tumor cells.

EGF and TGFα, natural ligands of the EGFR, have been shown to stimulate the proliferation of breast cancer cells, and the proliferation and morphogenesis of the normal MEC from which this cancer is derived. Surgical removal of the salivary gland which eliminates the main source of circulating EGF, or treatment with a neutralizing α-EGF antibody was shown to inhibit the development of spontaneous mammary tumors, the growth of established mammary tumors, and the implantation of transplantable mammary tumors in mice (2). Administration of EGF was able to reverse these effects (2). TGFα has been shown to mediate the mitogenic effects of estrogen, progesterone and prolactin in breast cancer cell lines (3), and part of the growth promoting effects of an activated ras gene in MEC (4). TGFα can also act as a dominant transforming gene product in MEC expressing normal levels of EGFR. Moreover, TGFα mRNA and protein can be detected in ~50-70% of primary human breast tumors (5).

An important perspective in understanding the mechanisms of growth control in cancer is the knowledge of growth regulation in the normal cells from which this cancer is

derived. A detailed understanding of the mechanisms of action, and the factors that control the type and/or magnitude of the response(s) induced by EGF and TGFα could be used to design effective forms of treatment that inhibit breast cancer progression. The successful design and implementation of curative therapies also requires an understanding of (1) the role that mammary gland stromal cells play in regulating the progression and metastatic spread of the breast cancer cells and (2) the mechanism of action and resistance to the proposed therapy. Thus far investigations in normal MEC have lagged behind studies examining the influence of growth factors in breast cancer because of the difficulties of supporting physiologically relevant development of normal MEC in culture. The main advantage of culture experiments is that experimental conditions can be controlled, thus allowing for more definitive interpretation of the results.

A unique and powerful primary culture system was developed in our laboratory which permits non functional MEC, isolated from pubescent female rats (at a time of development when these cells are maximally sensitive to carcinogen-induced transformation), to undergo extensive physiologically relevant proliferation, functional differentiation, and branching alveolar morphogenesis. This model system is uniquely suited to examine the mechanism(s) by which EGF and TGFα, acting through their common receptor, the EGFR, regulate the *in vitro* growth, differentiation, survival of (1) primary MEC derived from normal female rats, as well as (2) primary MEC isolated from rats exposed to the carcinogen N-methyl-nitrosourea (NMU). Future studies will culture normal as well as carcinogen-exposed MEC in the absence or in the presence of a phorbol ester tumor promoter known to synergize with certain activities of EGF and TGFα in MEC.

Our model system was also modified to permit MEC to be cultured in combination with different populations of mammary stromal cells. This modification has allowed for the examination of direct epithelial-specific effects when the MEC are cultured without mammary stromal cells, and of direct and indirect effects when the MEC are co-cultured with different types of mammary stromal cells. Development of a defined serum-free primary mammary co-culture system represents a significant advance for research aimed at examining stromal-epithelial interaction during normal MEC development as well as breast cancer progression and metastasis.

B.   BACKGROUND

The normal mammary gland undergoes its most extensive development during puberty and again during pregnancy and lactation. This type of development, referred to as branching morphogenesis, involves the precise regulation of cell proliferation, differentiation, apoptosis, as well as invasion, and leads to the formation of morphologically and functional distinct organs including the salivary gland, liver, lung, and kidney as well as the mammary gland. Mammary epithelial cells (MEC) undergo branching morphogenesis in response to the coordinate presence of distinct extracellular matrix (ECM) components including laminin, type IV collagen, entactin, fibronectin, and glycosaminoglycans (6), and hormones derived from the pituitary, adrenal, ovarian, and salivary glands (2,7-11). Induction and maintenance of this process is dependent on the differential ability of the epithelial cells to produce, degrade, and/or activate mammary gland parenchymal and mesenchymal cell regulators, and to adjust their polypeptide, steroid and ECM receptor status (12-16). Currently, the MEC-, and the mesenchymal cell-specific mechanisms of action of the systemically and

locally derived regulators of MEC branching morphogenesis, and their interactions are incompletely understood.

One of the most basic phenotypic differences between breast cancer cells, and the normal epithelial cells from which this cancer is derived appears to be the atypical morphology of the cancer cells compared to the highly specialized and well polarized appearance of the normal cells. Breast cancers in humans and rats generally develop within the highly proliferative and invasive end buds, developing alveolar buds, and along the ducts of the mammary gland (17-22). The distinct histologic types of breast cancer (23,24), and the late stages at which this disease is often diagnosed in humans have made it difficult to identify and characterize all of the factors that initiate and/or promote either the conversion of a normal cell to a pre-cancerous cell, or the progression of a pre-cancerous lesion to a carcinoma *in situ*, and finally to an invasive carcinoma. The appearance of morphologic atypia indicates a deregulation of normal branching morphogenesis. The factors which stimulate proliferation and invasion of normal MEC seem to also stimulate breast cancer progression and metastasis. These growth factors and their receptors are, therefore, viable therapeutic targets. A detailed understanding of the regulators of normal branching morphogenesis is required if these therapeutic strategies are to effectively inhibit breast cancer cell growth and metastasis.

1.   EPIDERMAL GROWTH FACTOR RECEPTORS (EGFR)

Basic EGFR Biology

The EGFR is a member of the type I receptor tyrosine kinase receptor family also known as the erbB or EGFR receptor family. The EGFR is a 170 kDa transmembrane glycoprotein with an extracellular domain for binding of EGF-like ligands such as EGF and TGFα. EGF-like ligands are characterized by a 3-loop secondary structure generated when 6 evenly spaced cysteine residues form disulfide bonds. The intracellular domain of the EGFR has a tyrosine kinase domain with an ATP-binding site, as well as binding sites for proteins that contain SRC homology 2 (SH2) or phosphotyrosine-binding domains (25-27).

Overview of EGFR Signal Transduction in Response to EGF

Upon binding EGF, the EGFR undergoes conformational changes in the extracellular domain that result in rapid oligomerization and intermolecular phosphorylation of occupied EGFR, followed by the association with and phosphorylation of kinases, phosphatases, adaptor proteins as well as other erbB receptors (26-37). EGFR activation has been shown to induce membrane hyperpolarization, probably by activation of K+ channels, alkalinization of the cell cytosol by increasing membrane Na+/H+ antiport activity, accumulation of intracellular calcium by indirect activation of protein kinase C, and amino acid and glucose transport (26,38). EGF has also been shown to stimulate the synthesis of type IV collagen (39), fibronectin (40), a 95 kDa type IV collagenase and interstitial fibroblast-type collagenase (41), to induce the expression of the EGFR (42), TGFα (42), and the cellular protooncogenes c-fos and c-myc (43), and to activate casein kinase II (44,45) which has been shown to phosphorylate DNA topoisomerase II (45), and the transcription factor myc (46). Recently, EGFR activation by EGF was shown to decrease the binding of ZPR1, a zinc

finger binding protein, to the intracellular tyrosine kinase domain of this receptor, and dissociated ZPR1 protein was then observed to translocate and accumulate in the nucleus.

EGFR Signal Tranduction in Response to TGFα

The signal transduction pathways induced by TGFα activation of the EGFR have not been studied as extensively as the intracellular consequence of EGF activation of the EGFR. It is interesting to speculate that each ligand initiates the induction of distinct signal transduction cascades within the different histologic types of normal MEC and/or breast cancer cells, and that these transduction pathways ultimately lead to the induction or suppression of distinct cellular responses.

EGFR Expression in Normal Mammary Glands

Both high (Kd of 0.1 nM) and low affinity (Kd of 3.6 nM) EGFR are present within cultured mouse MEC (47), and mammary glands from young and mature virgin, pregnant and lactating mice (48). Competitive *in situ* binding assays and autoradiography in the mammary glands of pubescent female mice were used to demonstrate that EGFR were concentrated in the cap cells of the terminal end buds, in the myoepithelial cells of the mammary gland ducts, and the stromal cells adjacent to the end bud flank and the subtending ducts (49). Immunohistochemical analysis of normal human breast and benign mammary tumors indicated that EGFR were usually expressed at a low level (50).

EGFR Expression in Breast Cancer

Changes in the expression and distribution of EGFR could play an important role in the pathogenesis of breast cancer. Analysis of EGFR in breast carcinomas using binding assays demonstrated an increased level of EGFR in 35-45% of the cases (51,52), and an inverse relationship with estrogen and progesterone receptors; in such tumors, a poor response to endocrine therapy, shorter disease-free period, and reduced overall survival, was noted. These tumors also have a higher proliferation rate (53). The increased levels of EGFR are generally due an increased level of EGFR mRNA expression (54,55). Gene amplification has only been observed in ~3% of the primary carcinomas studied (55).

In situ hybridization for EGFR mRNA shows that there generally is a good correlation with immunohistochemically detectable EGFR protein, but there are tumors in which EGFR mRNA can be detected in the absence of EGFR protein (56). In fact, EGFR transcripts are overexpressed in ~50-60% of primary human breast tumors (1). Data from breast cancer cell lines suggests that increased expression alone is not sufficient to produce hormone or growth factor independence (57).

EGFR-mediated induction of cell proliferation and invasion requires its interaction with an EGF-like peptide. Therefore, EGFR overexpression in the absence of an EGF-like mitogenic peptide cannot be expected to promote cancer progression. Transfection studies in rodent fibroblasts demonstrated that overexpression of the EGFR can predispose cells to expression of a transformed phenotype upon stimulation by EGF (58-60). In summary, deregulation or upregulation of EGFR levels, affinity and/or activity would be expected to provide the tumor cells with a ligand-dependent growth advantage. Finally, overexpression

of EGFR has been described in large numbers of human breast cancers, and is usually a poor prognostic indicator (1,51,61-67).

2.   EGF AND TGFα

Effects of EGF in Normal MEC

EGF has been shown to stimulate the proliferation and occasionally the differentiation of cells within the kidneys, lung, bone, brain, and skin, to affect hormone production in the hypothalamus, pituitary, placenta, ovaries, adrenals, and thyroid gland, as well as to modulate the immune system (38). Our laboratory and others have demonstrated that EGF is a critical physiologically relevant regulator of the growth, differentiation, and morphogenesis of normal MEC in culture as well as *in vivo*. Specifically, EGF stimulates cell proliferation, and supports MEC branching morphogenesis. EGF has also been shown to inhibit (47,68-73), enhance (2,74), or differentially regulate (75,76) milk protein production and/or expression of distinct milk components. The exact role that EGF plays in inducing and/or maintaining the cytological differentiation of these cells, and the mechanism(s) of action of EGF in mammary cells remains to be determined.

Effects of TGFα in Normal MEC

TGFα, another member of the growing family of EGF-like peptides, has also been shown to be a physiologically relevant regulator of mammary gland morphogenesis. The biological activities of TGFα have not been studied as extensively as that of EGF, and its mechanism(s) of action in mammary cells are largely unexplored. Administration of exogenous EGF or TGFα in Elvax pellets *in vivo* stimulated end bud and ductal growth (end bud branching morphogenesis) in the mammary glands of virgin ovariectomized mice (77). EGF and TGFα also enhanced lobuloalveolar development in hormonally primed virgin mice (78). TGFα has been shown to be secreted by rat mammary myoepithelial cells and epithelial cell lines in culture, as well as in the rat mammary gland were it can act as an autocrine and/or paracrine growth factor (79).

Expression of EGF and TGFα in During Mammary Gland Development *In Vivo*

Mammary glands from virgin (pubescent and adult) and mid-pregnant mice were shown to express both EGF and TGFα mRNA transcripts using reverse transcription-polymerase chain reaction (77). In contrast, mammary glands of mid-lactating mice only expressed EGF transcripts (77). Using conventional Northern blot analysis, TGFα mRNA was detected within mammary glands of pregnant rats, but not in mammary glands of virgin or lactating female rats (79).

Interestingly the concentration of TGFα in mammary gland extracts from virgin and pregnant rats was 0.2 ng/g of tissue, but in the lactating mammary gland the concentration was 1.2 ng/g of tissue (79). In contrast, TGFα expression is enhanced in mammary glands of humans during pregnancy and lactation, which may account for the relatively high level of this growth factor in human milk (80).

Immunohistochemical localization studies in prepubescent mouse mammary glands demonstrated that EGF was localized in the inner layers of the terminal end bud, and in the ductal cells of the mammary gland (77). These cells tend to be rather dominant, and cytologically differentiated. In contrast, TGFα was localized in the epithelial cap-cell layer of the advancing terminal end bud, and the stromal fibroblasts at the base on the highly proliferative and invasive terminal end bud. The peripheral cap cell layer is considered to be a population of proliferative stem cells. TGFα is also expressed in rat and human mammary glands in 10-15% of the surrounding stromal cells, as well as in both alveolar and ductal epithelial cells (80). Immunohistochemical studies in the mammary glands from rats during lactation localized EGF to the luminal surface of the secretory cells (81).

Summary of the Effects and Expression of EGF and TGFα during the Development of Normal Mammary Cells

Taken together, EGF may be responsible for stimulating moderate cell growth of normal MEC, but may play a primary role in inducing and/or maintaining differentiation of luminal epithelial cells. Endogenous TGFα may play a key role in the local regulation of stem cell proliferation during end bud branching morphogenesis by autocrine and paracrine mechanisms, and in the maintenance of functional differentiation during pregnancy and lactation. EGF appears to be derived primarily from the salivary gland as well as from the MEC, whereas TGFα is derived from both mammary gland parenchymal and mesenchymal cell types. Mammary expression of both EGF and TGFa appear to be hormonally- as well as developmentally-regulated.

Remaining Questions Regarding the Roles of EGF and TGFα in Mammary Gland Biology and Neoplasia

It seems clear that the current data does not thoroughly define the specific roles that the different EGFR ligands play in regulating the distinct stages of mammary gland development. Little is know about the role that EGF or TGFα play during the extensive alveolar regression that occurs during mammary gland involution. Comparative analysis of the biological activities of EGF and TGFα is required to determine whether each polypeptide plays distinct or similiar roles in regulating normal mammary gland morphogenesis. If TGFα is a preferential inducer of normal end bud proliferation and invasion, it may also play a similar role in breast cancer by promoting tumor cell growth and metastasis.

Additional immunohistological localization studies of EGFR and its ligands during the different stages of mammary gland morphogenesis should provide in-depth insight into the distinct roles that EGFR, EGF and TGFα play in the different phases of MEC branching end bud morphogenesis during puberty, MEC branching alveolar morphogenesis during pregnancy and lactation, epithelial apoptosis during involution, and neoplastic progression. It is also of interest to directly compare the type of signal transduction pathways that are induced in response to EGF or TGFα within mammary gland ducts, end buds, and alveoli, as well as pre-cancerous lesions, carcinoma *in situ*, and invasive carcinoma.

Effects and Expression of EGF and TGFα during Mammary Tumorigenesis

EGF and TGFα have both been implicated as factors which can stimulate the proliferation of various types of tumors. Both EGF and TGFα have been shown to be potent mitogens for breast cancer cells (1,82-88). Since the salivary gland is the dominant source of biologically active EGF in the body, Oka and co-workers surgically removed the salivary glands of certain mice to examine the role that salivary gland-derived EGF plays in mouse mammary tumorigenesis (2). The incidence of spontaneous mammary tumors in control virgin mice was ~63%. Sialoadenectomy reduced the tumor incidence to ~13% and increased the latency period of tumor development. These effects were partially reversed by administration of EGF. Sialoadenectomy of mammary tumor-bearing mice caused a rapid and sustained inhibition of tumor growth, whereas EGF stimulated tumor cell growth. Implantation of mammary tumor transplants was completely inhibited by sialoadenectomy. In addition to sialoadenectomy, the administration of anti-EGF antiserum inhibited the growth and implantation of mammary tumors (2). EGF treatment was able to reverse or block these effects.

TGFα mRNA can be detected in both benign and malignant breast tumors at a similar frequency and level of expression (89). Immunohistochemical studies revealed minimal levels of TGFα in normal non-parous human breast tissue, and increased levels in ductal hyperplasia, atypical ductal hyperplasia and ductal carcinoma *in situ* (90). TGFα mRNA expression and immunoreactive TGFα have been found in 40-70% of primary and metastatic human breast tumors (5). It is predominantly expressed in the tumor cells, not in the surrounding stromal cells or in the infiltrating lymphoid cells, and is generally expressed at a level that is significantly higher than in benign breast lesions, or in adjacent, non involved breast epithelium. Transformation of the spontaneously immortalized non transformed mouse mammary epithelial cell lines, NOG-8 and HC-11, and a human mammary epithelial cell line, MCF-10A by an activated c-Has-ras protooncogene increased the level of TGFα mRNA and protein expression by 5-10 fold (5). Addition of an anti-TGFα neutralizing monoclonal antibody to the ras-transformed MEC inhibited anchorage-independent growth of these cells by 50-80% which indicated that TGFα mediates at least part of the mitogenic effects of an activated ras gene in MEC. Overexpression of TGFα can act as an oncogene in NOG-8 mouse cells, and human MCF-10A cells (5,91). These cells have previously been shown to express EGFR. Introduction of rat or human TGFα into the germ line of transgenic mice induces abnormal morphogenesis and proliferation of the mammary glands.

C.   PURPOSE OF THE PRESENT WORK

Overview

A number of polypeptides and steroids have been identified that stimulate the development and/or progression of breast cancer. Specifically, EGF and TGFα have been shown to stimulate breast cancer cell proliferation, survival and invasion. Studies to directly compare the biological activities of EGF and TGFα in normal MEC are in their infancy. In addition, the regulators that control the type, magnitude and duration of responses induced by EGF or TGFα in normal MEC, or breast cancer cells have not been fully explored. Preliminary data from other laboratories suggests that EGF and TGFα play distinct roles during mammary gland development. TGFα may play a dominant role as a mitogen for epithelial stem cells and a secondary role in maintain epithelial differentiation during lactation.   In contrast, EGF may play a dominant role in inducing and maintaining the

cytologic as well as functional differentiation of MEC, and a secondary role in stimulating epithelial cell growth.

Background Data

Our data indicates that EGF stimulates cell proliferation [0.1-100 ng/ml], branching alveolar morphogenesis [1-100 ng/ml], and functional differentiation [1-100 ng/ml] (74). The effect of EGF on organoid invasiveness has not been quantified. Our data has also shown that at low concentrations [0.1 ng/ml], mouse EGF (mEGF) appears to selectively stimulated epithelial cell growth without having any affect on morphogenesis or functional differentiation. The effects of EGF on the cultured MEC were only observed when primary MEC were simultaneously cultured within a reconstituted basement membrane rich in laminin and type IV collagen as well as in medium that contained insulin, prolactin, progesterone, hydrocortisone, transferrin , bovine serum albumin and ascorbic acid (74,92).

Role of EGFR- and TGFα-Expressing Mammary Stromal Cells in the Development of Normal Mammary Epithelial and Breast Cancer Cells

Mammary gland stromal cells that naturally surround developing normal MEC as well as breast cancer cells have been shown to express EGFR as well as TGFα. The roles that EGFR positive and/or TGFα–expressing mammary gland stromal cells play in the normal development of MEC, and the progression and/or metastasis of breast cancer cells are presently unclear. A comprehensive understanding of all of the regulators of MEC and breast cancer development is essential for the development of effective prevention of and curative therapies for breast cancer.

Regulation of EGFR Biology and Functional Activity

Since EGF and TGFα induce their cellular responses through the EGFR, changes in EGFR expression and intracellular localization of EGFR during branching alveolar morphogenesis would be expected to influence the type and/or magnitude of responses observed when MEC were exposed to either of these ligands. It is important to determine whether the response to EGF or TGFα in rat MEC is determined by the location and number of the EGFR, or by co-expression of the other erbB receptors. Reports from other laboratories have shown that the binding activity, kinase activity, and internalization of the EGFR are regulated by autophosphorylation on tyrosine residues, other protein kinases that phosphorylate the receptor on specific threonine/serine residues, and by tyrosine-specific phosphoprotein phosphatases (93). Such tight regulation suggests that EGFR expression, intracellular localization, EGFR dimerization with EGFR, erbB2, erbB3 or erbB4, as well as EGFR tyrosine kinase activity are hormonally and developmentally regulated. Characterization of such regulatory mechanisms would expand the present understanding of the role that growth factor receptor tyrosine kinases play in normal development, and might explain why these receptors appear to be important in the pathogenesis of many forms of cancer including breast cancer.

Clinical Relevance

Although EGF and TGFα have been shown to stimulate the growth of a variety of breast cancer cells (2,94-97), it is not clear whether these ligands play synergistic or antagonizing roles in regulating breast cancer progression. It is interesting to speculate that there are differences in the type and/or magnitude of the responses induced by these growth factors in normal MEC compared to carcinogen-exposed and/or tumor-promoted MEC. Since both of these growth factors have been shown to stimulate metalloproteinase secretion (41,98) and cell migration (99-101), it is possible that they promote local invasion of normal MEC as well as breast cancer cells and/or metastasis of breast cancer cells. EGF transcripts and protein were detected in a majority of the commonly used breast cancer cell lines as well as in many (15-83%) primary human invasive breast cancers (61). Overexpression of TGFα has already been described in 40-70% of primary and metastatic human breast cancers suggesting the importance of understanding the functions of the EGF-like peptides in normal and malignant MEC (102). Overexpression of TGFα in transgenic mice caused epithelial hyperplasia, carcinoma *in situ* as well as adenocarcinomas in the mammary gland (103,104). Deregulation or upregulation of EGFR levels, affinity and/or activity can provide breast cancer cells with a ligand-dependent growth advantage. Moreover, high proportions of primary human breast cancers not only overexpress EGFR, one of the EGF-like ligands, and/or another erbB receptor, but these tumors usually respond poorly to endocrine therapy due to a lack of functional estrogen and/or progesterone receptors, and the patients generally have a short disease-free period and reduced overall chance for long term survival (1,51,61-67).

D.   METHODS OF APPROACH

Aim 1. Studies were designed to compare the biological activity of exogenous EGF and TGFα on the *in vitro* development of normal MEC cultured within a complex RBM in the presence of defined serum-free medium. This aim focused on evaluating the ability of both of these growth factors to modulate cell proliferation, differentiation, and apoptosis with the goal of determining whether these two growth factors play distinct or similar roles in regulating the *in vitro* development of normal MEC.

Aim 2. This aim was initially designed to compare the biological activity of exogenous EGF and TGFα in MEC isolated from mammary glands of normal rats compared with rats injected with the carcinogen N-methyl-nitrosourea (NMU). The goal of this aim was to determine whether the type and/or the magnitude of the effects induced in normal cells were different than observed in carcinogen-exposed cells, and to evaluate whether either or both of these polypeptides enhanced tumor progression *in vitro* in the absence as well as in the presence of a phorbol ester tumor promoter.

Aim 3. Studies were designed to examine the level and intracellular localization of EGFR within mammary glands from rats during different stages of development as well as within MEC cultured under conditions that induced branching alveolar morphogenesis. The goal of these studies was to determine whether there was a direct link between the level and/or intracellular localization of EGFR and the type of function (mitogenic, lactogenic, survival and/or apoptotic) induced in the MEC.

Aim 4. Studies were designed to examine the level and intracellular localization of EGFR within MEC isolated from rats injected with the carcinogen NMU as well as within pre-malignant and malignant lesions that developed within the mammary glands of rats injected with the carcinogen NMU. Comparison of these profiles with those obtained from normal MEC undergoing branching alveolar morphogenesis *in vivo* and *in vitro* should help to define the relationship between EGFR levels and/or intracellular localization and the development and progression of carcinogen-induced mammary tumors.

Aim 5. Studies were designed to compare the functional consequence of EGF- and TGFα-activation of the EGFR within MEC from normal compared with carcinogen-exposed rats. The objectives of these studies were to determine whether these two growth factors activate distinct or similar signal transduction pathways during normal branching alveolar morphogenesis *in vitro*, and whether EGFR signaling in normal MEC differs from that with MEC from carcinogen-exposed rats.

BODY

A.   **AIM 1. DEFINE THE ROLES OF EGF AND TGFα IN REGULATING THE *IN VITRO* DEVELOPMENT OF NORMAL MEC IN PRIMARY CULTURE.**

1.   **OVERVIEW**

A variety of experiments were undertaken to examine and compare the biological effects of EGF and TGFα on the proliferation, differentiation, and apoptosis of normal rat MEC. It was hoped that this information would provide a deeper understanding of the roles that each of these growth factors play in regulating the normal developmental processes of branching morphogenesis. During branching morphogenesis, normal epithelial cells proliferate, differentiate, undergo apoptosis, and migrate as the epithelium naturally invades the surrounding stromal tissue in a precisely controlled and highly conserved organ-specific pattern. Interestingly, breast cancer cells may retain this locally invasive characteristic. This invasive predisposition may explain why this type of cancer progresses into such a deadly disease.

We also hoped that this information would provide a solid baseline for comparative studies between normal MEC and breast cancer cells (that is carcinogen-exposed and/or tumor-promoted MEC). Thus far, EGF and TGFα have been found to exert a wide variety of biological effects in the normal rat MEC and appear to regulate such distinct cellular processes as proliferation, differentiation, morphogenesis, as well as apoptosis. Surprisingly, however, the type and magnitude of the effects induced by EGF and TGFα have in large part been identical (see annual reports for grant periods from July 1, 1994 - June 30, 1995 and from July 1, 1995 -June 30, 1996) despite the preliminary results reported by other laboratories that suggested that the biological activities of these two growth factors were distinct. It should be emphasized that our studies were unique in that they were carried out using MEC isolated from normal pubescent female rats and these primary cells were cultured under defined serum-free conditions. These culture conditions were previously shown to enable primary MEC to retain *in vivo* relevant cellular responsiveness to steroids, as well as polypeptide hormones, growth factors, cytokines and extracellular matrix components (74,92,105-107).

Two years ago, our laboratory obtained from Parke-Davis a potent and selective inhibitor of the tyrosine kinase domain of all of the members of the ErbB receptor family including the EGFR. This inhibitor, PD158780 (108), was used in a number of different types of primary culture and cell culture studies. This drug provided us with a powerful and effective way of specifically turning off ErbB receptors including EGFR (personal communication with Dr. David Fry at Parke-Davis as well as data presented in Aim 5). As such, availability of drugs like PD158780 represent a dramatic advance for studying EGFR and ErbB receptor biology as well as in developing pharmacologic approaches to regain control of cells that overexpress EGFR and/or their ligands.

Thus far, PD158780 has allowed us to (1) more accurately identify the role(s) that endogeneous EGFR ligands play in regulating the *in vitro* development of MEC cultured in the absence of exogenously supplied EGF or TGFα, and (2) define the specific role(s) that functional EGFR plays during the *in vitro* development of MEC when these normal epithelial

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cells were cultured under conditions that promoted branching alveolar morphogenesis. Although initial hopes were to examine the effects of EGFR ligands on branching alveolar morphogenesis as well as branching end bud morphogenesis, these primary culture studies were extremely large and technically challenging to carry out and analyze. Attentions were, therefore, focused on evaluating the effects of EGF and TGFα on the proliferation, functional differentiation and apoptosis of MEC under conditions of branching alveolar morphogenesis. It should be noted that although a number of specific figures presented within the last two annual reports covering the grant periods from July 1, 1994 - June 30, 1995 and from July 1, 1995 -June 30, 1996 do not appear in this final report, the data presented within this report summarizes the research findings of the entire grant effort.

2. EXPERIMENTAL METHODS

Isolation of Mammary Epithelial Cells (MEC)

Abdominal and inguinal mammary glands excised from pubescent virgin female (50- to 60-day-old) Sprague-Dawley rats were mechanically and enzymatically disaggregated. The pooled mammary glands were minced into small fragments and then cultured at 37°C in digestion medium [10 ml/g] (0.2% [w/v] collagenase type III, 0.2% [w/v] dispase grade II, 5% newborn calf serum and 50 µg/ml gentamycin in PRF RPMI 1640). Mammary glands from virgin rats were digested for ~14.5 hours. The digest was fractionated by centrifugation (500 x g for 10 min) and pelleted cells were suspended in PRF-RPMI 1640. The suspension was filtered through a 530 micron nitex filter, and the resulting eluate was passed through a 60 micron nitex filter. This filtrate was discarded whereas the organoids [organ-like fragment] retained on the filter were washed off with a 1:1 mixture of PRF F12/DMEM containing 5% [v/v] newborn calf serum and 50 µg/ml gentamycin. Fibrous debris that appeared during the filtration steps was then removed by a second 530 micron nitex filtration. The isolated organoids were then cultured for 4 h at 37°C to allow for the attachment and subsequent removal of mammary gland fibroblasts. The number of cells within the organoid suspension was estimated from triplicate nuclei counts as previously described (109). The non-adherent organoids obtained from pubescent virgin rats were cultured (as described below) within a reconstituted basement membrane (RBM) prepared from the Engelbreth-Holm-Swarm sarcoma (74).

Primary Culture Conditions

Non-adherent organoids were carefully suspended in ice cold RBM at a density of 1.5 x 10^6 or 4 x 10^6 cells per ml of RBM. Using standard 24 well tissue culture plates, 200 µl of organoid-RBM suspension containing approximately 3 x 10^5 or 8 x 10^5 cells was plated on top of 200 µl of pre-solidified RBM within each culture well. Alternatively, 2.5 ml of organoid-RBM suspension containing approximately 1 x 10^7 cells was plated on top of 2.5 ml of pre-solidified RBM when 100 mm tissue culture dishes were used. The matrix suspension was allowed to solidify at 37°C for 3.5-4 hours before 1 ml or 12.5 ml of the appropriate type of culture medium was added to each culture well or 100 mm dish, respectively.

Culture medium was changed every 3.5 days throughout the 21 day experiment. For the studies described in this report, the cells were cultured with defined serum-free medium

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referred to as ALV Medium. ALV Medium represented F12/DMEM medium with bovine insulin [10 µg/ml], ovine prolactin (NIDDK o-PRL-17, -19, or -20) [1 µg/ml], progesterone [1 µg/ml], hydrocortisone [1 µg/ml], human apo-transferrin [5 µg/ml], ascorbic acid [880 ng/ml], fatty acid-free BSA [1 mg/ml], and gentamycin [50 µg/ml]. The MEC were cultured for 3.5, 7, 10.5, 14, 17.5 or 21 days in ALV Medium alone (NoGF medium), ALV Medium with 10 ng/ml human recombinant EGF (EGF medium), or ALV Medium with 10 ng/ml human recombinant TGFα (TGFα medium).

Primary culture studies were carried out to define a non-cytotoxic and effective. In these studies, primary MEC were cultured for up to 21 days in the continuous presence of NoGF medium with 0, 0.05, 0.5 or 5 µM PD158780, EGF medium or TGFα medium. The latter two culture groups were not exposed to PD158780 during the study. PD158780 was added to the appropriate type of medium just prior to the media change from a 10 mM stock in 100% DMSO that was stored at -80°C. Each of the different types of media used in these studies contained a final concentration of 0.1 % (v/v) dimethylsulfoxide (DMSO). For these primary culture studies, the non-adherent organoids were isolated from mammary glands pooled from six to twelve pubescent virgin rats. Each treatment condition was carried out in at least two and as many as eight different experiments.

Other primary culture studies utilized a short term exposure period of MEC to 0.5 µM PD158780. It was hoped that exposure of MEC to PD158780 for a 3.5 day period of time would selectively turn off erbB receptor signaling (including EGFR signaling) during this interval and thus allow the evaluation of the direct consequence of knocking out EGFR function. MEC were cultured in the presence of PD158780 for the last 3.5 days of a 3.5-, 7-, 10.5-, 14-, 17.5- or 21-day experiment. MEC were exposed 0 or 0.5 µM PD158780 in 0.1% (v/v) DMSO. PD158780 was added to the appropriate type of medium just prior to the media change from a 10 mM stock in 100% DMSO that was stored at -80°C. For these primary culture studies, the non-adherent organoids were isolated from mammary glands pooled from six to twelve pubescent virgin rats. Each treatment condition was carried out in at least two and as many as eight different experiments.

In each of these experiments, viable cell number was monitored using a tetrazolium dye assay whereas viable colony number was evaluated by counting the number of purple (tetrazolium-stained) colonies within individual culture wells using a CK-2 Olympus microscope. MEC-functional differentiation was assessed either using an enzyme-linked immunoabsorbant assay to quantitate the total level of casein protein accumulated within individual culture wells or using a Western blot procedure to monitor casein isoform expression (casein milk proteins represent a large family including α_1, α_2, β, γ, and κ casein). Morphologic evidence of epithelial apoptosis was evaluated by light microscopic examination of the individual epithelial organoids (colonies), electron microscopic examination of representative colonies exhibiting light microscopic evidence of apoptosis, as well as using an *in situ* terminal deoxynucleotide transferase (TUNEL) assay.

Tetrazolium Dye Assay to Quantify Viable Cell Number

Specific changes in the number of viable cells was evaluated using a 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) assay (105,110). Briefly,

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cultures were incubated with MTT at a final concentration of 1 mg/ml for 16 hr at 37°C. The RBM was digested away from the MEC using 5 units/ml grade II dispase in F12/DMEM medium. The aqueous-insoluble formazan crystals were collected, washed and solubilized in 2-propanol. Sample absorbance was read with a Bio-tek EL-311 automatic plate reader at 570 nm. Production of formazan crystals and the absorbance of the solubilized crystals was directly proportional to viable cell number. A standard curve was set up with the newly isolated MEC for each experiment.

Enzyme-Linked Immunosorbant Assay to Quantify Total Casein Accumulation
(Casein ELISA were run by Suzanne Shoemaker)

Casein accumulation, used as an indicator of MEC functional differentiation, was monitored using a previously described non-competitive enzyme linked immunosorbant assay (ELISA) with a rabbit anti-rat casein polyclonal antibody (109,110). Casein levels were quantified in triplicate culture wells per treatment type for each of the different time points. The RBM with and without MEC in individual culture wells was harvested with 600 μl of ice-cold, 1% (v/v) triton X-100 lysis buffer [150 mM sodium chloride, 50 mM Tris pH 8.0, 2 mM ethylenedinitrilotetraacetic acid (EDTA), 10 mM sodium phosphate, 10 mM sodium pyrophosphate, 5 mM sodium orthovanadate, 0.1% (w/v) sodium dodecylsulfate, 0.5% (w/v) sodium deoxycholate, 0.1 mM phenylmethyl-sulfonyl fluoride (PMSF), 100 ng/ml soybean trypsin inhibitor and 20 ng/ml leupeptin]. The harvested organoids plus RBM samples were sonicated on ice for 15 seconds using a Tekmar Sonic Disruptor (Cincinnati, OH). The samples were then centrifuged at 12,000 x g for 15 min at 4°C. The resulting supernatants were stored at -20°C. The total level of casein within each culture sample was measured using the casein ELISA with a phosphate-buffered saline (pH 7.4) buffer system. Each individual culture sample was assayed in duplicate or triplicate at four to six dilutions. Nunc immunosorbant plates were processed using a Bio-tek automatic plate washer, and sample absorbance analyzed at 405 nm using a Bio-tek EL-311 automatic plate reader.

Western Blot Procedure to Evaluate Casein Isoform Expression
(Ann Wohlhueter assisted in carrying out the casein Westerns.)

Casein protein expression was examined in whole cell lysate samples harvested from triplicate culture wells per treatment type for each of the different time points. Each whole cell lysate sample was prepared after the culture medium within each well was aspirated away and the MEC embedded within the RBM solubilized within ice-cold 1% (v/v) triton X-100 lysis buffer. Ice-cold lysates were then sonicated using 3x10 sec bursts of a Tekmar sonic distruptor set at 80% output, and then centrifugated for 15 minutes at ~12,000 x g. Lysate supernatants were then mixed with a reducing and denaturing sample buffer and then samples representing an appropriate number of cells separated on a 4-20% SDS-polyacrylamide gradient gel. Proteins were then electrophoretically transferred for 2 hours to an Immobilon P membrane. Membranes were then (1) blocked overnight at 4°C in phosphate buffered saline (PBS) at pH 7.3 containing 0.1% (v/v) Tween 20 and 5% (w/v) powdered instant milk, (2) washed several times with PBS containing 0.1% (v/v) Tween 20, (3) incubated for 90 minutes at room temperature with a 1:1 mixture of the rabbit anti-rat casein polyclonal 877 and 878 antibodies diluted 1:2000 in PBS with 0.05% (v/v) Tween 20 and 3% (w/v) bovine serum albumin, (4) washed several time with PBS containing 0.1% (v/v) Tween 20, (5) incubated for 60 minutes at room temperature with a 1:5000 dilution of an

affinity purified donkey anti-rabbit IgG antibody that was preabsorbed against rat, human, and mouse serum proteins and conjugated to horseradish peroxidase [Jackson Immunoresearch Laboratories Inc.], and (6) washed several time with PBS containing 0.1% (v/v) Tween 20. Immunoreactive proteins were visualized using an enhanced chemoluminescent reagent [Amersham], and X-ray films exposed in a dark room were developed using an automatic developer.

Microscopy, Photography and Apoptosis Detection
(Jennifer Black, Ann Wohlhueter and/or Mary Vaughan assisted with these evaluations)

An Olympus CK2 microscope mounted with a Nikon FX-35A camera was used for time-lapse light microscopic examination and photography of individual colonies. At the various times indicated in the text, three culture wells of living organoids for each treatment group were repeatedly examined to identify changes in colony number, size, coloring and/or shape during the course of the 21-day culture period. Cytological analysis of the cellular composition, organization and degree of differentiation within intact colonies cultured within the RBM was performed as follows. MEC within the RBM were glutaraldehyde-fixed, embedded within Spurr's resin, sectioned at ~90 nm, stained with uranyl acetate and lead citrate and analyzed using Siemens Elmiskop 101 electron microscope. Alternatively, cultured MEC were fixed for at least ~24 hours in 10% (v/v) phosphate-buffered formalin, processed in a lens paper tea bag overnight in an automatic tissue processor, embedded in paraffin, and sectioned at 5 μm using an AO Rotary microtome. Sections were collected onto silane-treated glass slides, deparaffinized in three changes of xylene, rehydrated, and processed using Oncor's ApopTag™ In Situ Apoptosis detection kit as per manufacturer's recommendations. Briefly, apoptosis is a multistep process of programmed cell death that results in fragmentation of DNA and the concentration of these 3'-OH DNA fragments within morphologically distinct condensed nuclei and apoptotic bodies. Digoxigenin-nucleotides were catalytically added to the 3'-OH ends of double or single stranded DNA within sections of the MEO by terminal deoxynucleotidyl transferase. An anti-digoxigenin antibody conjugated to peroxidase and the chromogen substrate DAB were then used to detect incorporated digoxigenin-nucleotides in the cultured MEO. An Olympus BH-2 microscope mounted with a Nikon FX-35A camera was used for photography of the hematoxylin-stained epithelial organoids evaluated using the In Situ ApopTag® kit.

Statistics
Data are presented as mean ± standard error of the mean (SEM= SD/$\sqrt{n}$). Statistical significance was evaluated using a one-way analysis of variance (ANOVA) with the Student-Newman-Keuls test for pairwise multiple comparisons. $P < 0.05$ was judged to be statistically significant.

3.   RESULTS and DISCUSSION

Our laboratory previously demonstrated that the proliferation, functional differentiation and morphogenesis of primary rat MEC was coordinately regulated by a complex group of polypeptides, steroids and extracellular matrix components (74,92,105,107,111). EGF was specifically shown to be a potent mitogen, lactogen as well as morphogen for primary rat MEC (74). The biological effects induced in response to EGF, however, were only observed when the MEC were cultured within the RBM in the presence of defined serum-free medium

supplemented with precise concentrations of insulin, prolactin, progesterone, hydrocortisone, transferrin, ascorbic acid and BSA (ALV Medium). The large number of co-regulatory factors required to induce specific effects in normal MEC as well as the asynchronous nature of MEC development have made it technically challenging to precisely define the type, magnitude, duration and/or mechanism of the EGFR-dependent effects in MEC.

DETERMINATION OF A NON-CYTOTOXIC AND EFFECTIVE CONCENTRATION OF PD158780 FOR NORMAL PRIMARY MEC

An initial primary culture study was carried out to select a concentration of PD158780, the pyrido-pyrimidine inhibitor of the ATP-binding site of the tyrosine kinase domain of the ErbB receptor family including the EGFR (108), that was non-cytotoxic in primary MEC. The isolated MEC were cultured within the RBM in the continuous presence of (1) EGF medium, (2) TGFα medium, or (3) NoGF medium supplemented with 0, 0.05, 0.05 or 5 μM PD158780. Epithelial cell number increased ~8-fold in the presence of EGF or TGFα medium (Figure 1A). In the absence of exogenous EGF, TGFα, or PD158780, epithelial cell number increased about 3-fold during the course of a 21 day study. PD158780 inhibited epithelial cell growth at all three concentrations (Figure 1A). PD158780 at 5 μM was, however, somewhat cytotoxic as demonstrated by the ~40% decrease in viable cell number (Figure 1A) and viable colony number (Figure 1B). PD158780 at 0.05 or 0.5 μM effectively inhibited epithelial cell growth (Figure 1A), but did not alter colony survival (Figure 1B).

The observation that non-cytotoxic concentrations of PD158780 were able to inhibit the growth of primary MEC in the presence of NoGF medium suggested that endogenous EGF-like ligand(s) were either released from the RBM and/or produced by MEC under these culture conditions. Laminin, tenascin and entactin, components of basement membrane surrounding the cultured MEC, contain numerous EGF-like repeats (61) that can be released during the proteolytic degradation of any of these ECM components. Furthermore, Vukicevic and co-workers demonstrated the presence of EGF as well as heparin-binding EGF within RBM prepared from the EHS-sarcoma (112). Our own preparations of RBM were shown to contain between 0.2 and 0.5 ng/ml EGF as demonstrated using a radioimmunoassay (74). Finally, a number of groups have demonstrated that normal MEC can produce a variety of EGF-like ligands including EGF and TGFα (77,80,81,113).

The next primary culture study confirmed that continuous exposure of MEC to 0.5 μM PD158780 effectively inhibited epithelial cell growth in the presence of medium containing 10 ng/ml EGF or TGFα (Figure 2). Specifically, the 12-fold increase in viable cell number observed when MEC were cultured for 21 days in medium supplemented with 10 ng/ml EGF or TGFα was almost completely inhibited by exposing the primary MEC to 0.5 μM PD158780 from day 0-21 of the study (Figure 2). In contrast, short term exposure of MEC to 0.5 μM PD158780 from day 17.5-21 did not affect the number of viable observed on day 21 of the experiment (Figure 2).

Rapid signal transduction experiments were then carried out to demonstrate that PD158780 at 0.5 μM selectively inhibited EGF-induced tyrosine phosphorylation in rat breast adenocarcinoma cells exposed to NoGF or EGF medium. The results of these studies are presented in Aim 5. It should be noted that attempts to detect rapid tyrosine phosphorylation

responses in cultured MEC failed primarily due to technical problems related to the disproportionately high levels of RBM proteins within these epithelial lysates.

EFFECTS OF EGF OR TGFα ON THE *IN VITRO* DEVELOPMENT OF PRIMARY MEC

MEC were then exposed to 0.5 µM PD158780 for short time periods during their *in vitro* development to determine when EGFR activation and signaling were required for the growth, survival, and/or functional differentiation of MEC. MEC were cultured within the RBM, in the presence of NoGF, EGF or TGFα medium for either 3.5, 7, 10.5, 14, 17.5 or 21 days. MEC within these groups were then exposed to 0 or 0.5 µM PD158780 during the last 3.5 days immediately prior to the end of the culture period. After the indicated number of days in culture, each of the experimental groups was analyzed for effects on viable cell number, apoptosis and casein accumulation to determine when the EGFR inhibitor was able to regulate for epithelial cell growth, survival and/or functional differentiation, respectively.

Mitogenic Effects of EGF and TGFα were
Preferentially Observed During the First Week of Culture

When cultured in the presence of NoGF medium, short term exposure of MEC to 0.5 µM PD158780 either had no effect on (at all time points in Figure 3A and at day 3.5 and 10.5 of culture in Figure 4A) or reduced (at day 7 in Figure 4A) the number of viable cells within individual culture wells. In these studies, endogenous EGF-ligands either played a minor role or were not involved in stimulating epithelial cell growth. Additional studies are required to isolate and characterize the mitogenic activity observed in these studies.

In the presence of EGF medium, short term exposure of MEC to 0.5 µM PD158780 from days 0-3.5 or days 3.5-7 of culture markedly inhibited epithelial cell growth (Figures 3A and 4A). In contrast, short term exposure of MEC to PD158780 during any time from day 7 through day 21 of culture did not affect epithelial cell growth (Figures 3A and 4A). In one experiment in which MEC were cultured in EGF medium, PD158780 exposure from days 10.5-14 moderately inhibited the growth of these cells (data not shown).

PD158780 exposure from days 0-3.5 (Figure 3A) or days 3.5-7 (Figures 3A and 4A), but not from days 7-10.5 (Figure 3A), days 10.5-14 Figure 4A), or days 14-17.5 (data not shown), inhibited epithelial cell growth in the presence of TGFα medium. Exposure of MEC cultured in TGFα medium to PD158780 from days 17.5-21 either did not affect (Figure 2) or inhibited (Figure 4A) epithelial growth.

These data demonstrate that EGFR signaling played a major role in stimulating epithelial cell growth during the first week of culture in EGF or TGFα medium, but not thereafter. Additional studies are required to determine the factors that dictate whether or not EGF and TGFα induce mitogenic responses in MEC. Furthermore, it will be worthwhile to determine which polypeptide(s) and/or steroid(s) assume the primary role in stimulating epithelial cell growth during the second and third weeks of culture in EGF or TGFα medium.

It should be noted that before PD158780 became available, primary culture studies were carried out in which MEC initially cultured in EGF medium were switched to NoGF medium in an attempt to terminate EGF-induced effects in the primary MEC by removing the

exogenous supply of this growth factor (74). Failure of this approach primarily reflected the difficulty of removing EGF trapped within the RBM after a 3.5, 7 or 14 day culture period in the presence of EGF medium.

EGF and TGFα Regulated the Survival of Terminally Differentiated MEC

It is important to keep in mind that the tetrazolium dye assay measures viable cell number which reflects the net balance between cell growth and death within a given culture well. Approximately 1-5% of epithelial organoids cultured for 21 days in the presence of EGF medium exhibit morphological signs of alveolar regression. MEC cultured for longer than 21 days progressively exhibit more dramatic morphological evidence of cell death within the epithelial colonies. Light microscopic analysis of regressing compared with healthy epithelial colonies revealed that the healthy colonies had a smooth surface and were composed of cells organized into lobular, multilobular as well as lobuloductal colonies (Figure 5A and 5C) (74,107). Electron microscopic examination of healthy epithelial colonies revealed that they were composed of viable, well polarized epithelial cells organized in either ductal or alveolar arrangements around distended central lumen that were often filled with morphologic evidence of milk protein and lipid (Figure 5E) (106,107). In contrast, apoptotic colonies literally appeared to be collapsing and breaking apart (Figure 5B and 5D). The cells along the outer surface of the colony began to pull away from the central mass (Figure 5D) and the surface of these colonies was often irregular and fuzzy in appearance. Electron microscopic examination of these colonies revealed the presence of condensed nuclear material, collapsed central lumen, little to no evidence of milk protein and an abundance of cytoplasmic lysozomes (Figure 5F). Alveolar regression of the epithelial organoids was shown to be associated with extensive programmed cell death as demonstrated by morphological criteria as well as *in situ* apoptosis detection using the TUNEL assay (Figure 5H compared to 5G). Furthermore, natural and retinoid-induced alveolar regression of the cultured MEC was also associated with classical DNA laddering (Shea and Ip, unpublished data).

Interestingly, most of the alveolar epithelial organoids that developed during the course of the three week culture period in either EGF or TGFα medium displayed extensive morphological evidence of apoptosis when the MEC were exposed to PD158780 from day 17.5 through day 21. However, morphological signs of alveolar regression were not observed when the MEC were cultured in EGF or TGFα medium and exposed to PD158780 during any time up until day 17.5. Futhermore, morphologic evidence of alveolar regression was not observed when MEC were cultured in NoGF medium and exposed to 0 or 0.5 μM PD158780 at any time during the 21 day study. These data suggest that EGFR signaling may regulate the survival of terminal differentiated alveolar MEC. It should be noted that at least four batches of RBM were produced that support the outgrowth of differentiated MEC that underwent apoptosis when exposed to 0.5 μM PD158780 from day 17.5-21. [One preparation of RBM, however, was deficient in this respect]. Taken together, these data suggest that the composition of the extracellular matrix is an important determinant in whether or not differentiated MEC undergo apoptosis in response to an inhibitor of EGFR (as well as ErbB2, ErbB3 and ErbB4 if expressed by the cultured MEC) signal transduction.

Follow up studies were then carried out to quantitate the apoptotic response observed when MEC were exposed to PD158780 starting on day 17.5 of culture. Histological analysis

of MEC exposed to PD158780 from days 17.5-21 (84 hour exposure) demonstrated that almost all of the multilobular alveolar organoids underwent extensive apoptosis and that this time point actually represents the final stages of the apoptosis process. *In situ* apoptosis detection using the TUNEL assay was carried out on formalin-fixed, paraffin-embedded MEC cultured with 0 or 0.5 μM PD158780 from days 17.5-18.5 (24 hour exposure) or from days 17.5-19.5 (48 hour exposure) and the number of apoptotic cells (visualized with the brown chromogen DAB) relative to the total number of cells within the individual sectioned organoids was quantified.

In the presence of NoGF medium, less than 3% of MEC within individual organoids contained apoptotic DNA in response to a 48 hour exposure to either 0 or 0.5 μM PD158780 starting on day 17.5 of culture (Table 1). When MEC were cultured in EGF medium and exposed to 0 or 0.5 μM PD158780 for 48 hours starting on day 17.5 of culture, less than 1% or ~40% of cells within individual colonies contained apoptotic DNA, respectively (Table 1). It should also be noted that only ~1% of MEC within individual colonies were apoptotic when MEC cultured in EGF medium were exposed to PD158780 from days 17.5-18.5 (24 hour exposure). Approximately 9% of MEC cultured in TGFα medium from days 0-19.5 contained apoptotic DNA whereas ~75% of the MEC within individual organoids contained apoptotic DNA when cultured from days 0-17.5 in TGFα medium and then in TGFα medium with 0.5 μM PD158780 for 48 hours (Table 1).

Interestingly, MEC cultured in NoGF medium compared to either EGF or TGFα medium were less functionally differentiated as indicated by their reduced capacity to accumulate casein milk protein (Figures 3B and 4B) which may explain why these cells did not undergo apoptosis when exposed to PD158780. Futhermore, MEC cultured in EGF or TGFα medium for at least 17.5 days, but not for shorter intervals, underwent apoptosis in response to PD158780. It should be noted that during the course of a 21 day culture period in the presence of EGF or TGFα medium, MEC progressively accumulated increasing quantities of casein milk protein (Figures 3B and 4B). Taken together, these data suggest that EGFR signaling is required for the survival of terminally differentiated, not simply functionally differentiated MEC. The delayed time course of the apoptotic response observed when MEC cultured for 17.5 days in either EGF or TGFα medium were exposed to 0.5 mM PD158780 (no apparent effect at 24 hours and a 40-75% response by 48 hours) may suggest that EGFR signaling induces *downstream* responses required to maintain the survival of terminal differentiated MEC. The apparent difference in the basal and PD158780-induced level of apoptosis observed in this experiment when MEC were cultured with TGFα rather than EGF will required follow up studies to ascertain biological significance.

EGF was also shown to be a survival factor for HC11 mouse mammary epithelial cell line (114), primary rat ventral prostatic epithelial cells (115), dental mesenchyme during tooth development in mice (116), transplanted hepatocytes (117), primary hepatocytes exposed to transforming growth factor β (TGFβ) (118), and primary human trophoblasts exposed to tumor necrosis factor α (TNFα) and interferon-γ (IFN-γ) (119). In contrast, EGF was shown to induce apoptosis in A431 (120), MDA-MB-468 human breast cancer cells (121) as well as in MTC (13762NF) rat mammary adenocarcinoma cell line expressing ectopic EGFR (122). Futhermore, overexpression of TGFα under the control of the whey acid protein (WAP) promoter in transgenic mice was shown to delay or inhibit mammary gland involution (the natural process whereby a majority of alveolar luminal epithelial cells

undergo apoptosis) (123,124). In addition, WAP-TGFα transgenic mice developed well-differentiated adenomas and adenocarcinomas with 100% incidence (123).

Additional studies are required to determine the mechanism by which inhibition of EGFR-dependent tyrosine phosphorylation induced epithelial apoptosis. Termination of EGFR signaling might directly induce apoptosis by shifting the ratio of cell death inducers (such as Bax and Bcl-x_S) and cell death protectors (such as bcl-2 and bcl-x_L). Bax and Bcl-x_S were both induced (125,126) whereas bcl-2 levels were decreased (126) during apoptosis of mouse MEC during involution. Furthermore, high levels of bcl-x_S and bcl-2 were preferentially expressed within apoptotic body cells in the core of terminal end buds during ductal morphogenesis of mouse mammary glands whereas bax was uniformly expressed in cells throughout the terminal end bud (127). Rodeck and co-workers demonstrated that inhibition of EGFR activity (using a neutralizing antibody or an EGFR-selective tyrosine kinase inhibitor) in human keratinocytes down-regulated the expression of Bcl-x_L and induced keratinocyte apoptosis (128). Hynes and co-workers demonstrated that in human mammary MCF-10A cells, EGF (a known survival factor), together with insulin, up-regulated bcl-2 levels (126).

The role that erbB2, erbB3 and/or erbB4 receptors play in regulating the survival of terminally differentiated MEC is currently unknown. It should be kept in mind that if terminally differentiated MEC expressed erbB2, erbB3 and/or erbB4, PD158780 would be expected to inhibit EGFR-dependent transactivation of erbB2, erbB3 and erbB4 as well as to inhibit the tyrosine kinase domain of the receptors themselves. Neuregulins (also called heregulins or neu differentiation factors) are the natural ligands for erbB3 and erbB4 (129-134). Recent studies have demonstrated that neuregulins regulate mammary epithelial cell growth, differentiation and branching alveolar morphogenesis (135). Thus far, neuregulins have been shown to regulate the survival of astrocytes (136), sympathetic neuroblasts (137), Schwann cells (138,139), and oligodendrocyte progenitors (140).

EGF and TGFα Regulated Casein Accumulation
within Functionally Immature and Apoptotic MEC

The role that EGFR activation and signaling plays in regulating the functional differentiation of MEC was evaluated by monitoring casein accumulation by the cultured MEC using an ELISA and a polyclonal antibody raised against the entire family of casein milk proteins (106). MEC cultured in NoGF, EGF or TGFα medium produced and accumulated increasing quantities of casein proteins (Figures 3B and 4B). As indicated above, MEC cultured in either EGF or TGFα medium accumulated more casein than MEC cultured without an exogenous supply of an EGFR ligand. When MEC were cultured in EGF medium, PD158780 exposure from days 0-3.5 (Figure 3B) or days 3.5-7 (Figures 3B and 4B), but not thereafter, effectively inhibited production and/or accumulation of casein (Figures 3B and 4B and data not shown). PD158780 exposure from days 0-3.5 (Figure 3B) or days 3.5-7 (Figures 3B and 4B) consistently inhibited casein production and/or accumulation when the MEC were cultured in TGFα medium, whereas exposure from days 7-10.5 or 17.5-21 either inhibited (Figures 3B and 4B, respectively) or had no effect (data not shown) on casein levels. EGFR signaling via EGF or TGFα appears to play a dominant role in stimulating casein production and/or accumulation during the first week of culture. This

data supports the hypothesis that EGF-like ligands are involved in the induction rather than the maintenance of functional differentiation in MEC.

EGF and TGFα Regulated Casein Isoform Distribution in Functionally Immature and Apoptotic MEC

Gene expression of the distinct casein isoforms is under the control of a complex array of transcription factors and repressors. Many of the casein isoforms are also glycosylated and/or phosphorylated. The functional significance of these post-translational modifications are not entirely clear. Western blot analysis was undertaken to determine whether EGFR plays a selective role in regulating casein isoform accumulation and/or post-translational modifications. MEC cultured for 3.5 days or 7 days in NoGF Medium accumulated α_1-, α_2 - and one form of β-casein (Figures 6 and 7). PD158780 exposure of the epithelial cells from days 0-3.5 inhibited the accumulation of all three isoforms (Figure 7). MEC exposure to PD158780 from days 3.5-7 either inhibited the accumulation of α_1-, α_2 - and β-casein accumulation (Figure 6) or preferentially increased the levels of the fastest migrating β-casein isoform (Figure 7). When cultured in NoGF medium for 10.5 days or longer, MEC accumulated α_1-, α_2-, κ-, multiple forms of β- and occasionally some γ-casein (Figures 6 and 7). In general, casein expression profiles were not affected by exposure of MEC to 0.5 μM PD158780 at any time after day 7 in culture (Figures 6 and 7).

When cultured in EGF or TGFα medium, MEC accumulated α_1-, α_2-, and one form of β-casein by day 3.5 (Figures 8 and 9, respectively) and α_1-, α_2-, κ-, a variety of forms of β- and occasionally some γ-casein thereafter (Figures 6, 8 and 9). Exposure of MEC to PD158780 from day 0-3.5 inhibited the accumulation of α_1-, α_2- and β isoforms (Figures 8 and 9) whereas exposure of MEC to PD158780 from day 3.5-7 inhibited α_2-, β- and κ-casein accumulation (Figures 6, 8 and 9).

These findings may suggest that during the first week of culture when MEC are just undergoing functional differentiation that EGF and TGFα play dominant roles in regulating casein transcription, the half-life of casein transcripts, casein synthesis, and/or the activity of enzymes involved in phosphorylation and/or glycosylation of β-casein. Additional experiments are, therefore, warranted to determine whether EGFR ligands are exerting a direct or indirect effect on casein accumulation. A direct mechanism may involve the activation of transcription factors known to specifically turn on β-casein gene expression, the secretion of extracellular matrix components known to activate transcription factors that upregulate β-casein gene expression, and/or to activate proteins like casein kinase II that can phosphorylate certain casein isoforms. Alternatively, an indirect mechanism may involve the inhibition of a protease(s) (such as a matrix degrading metalloproteinase that is know to exhibit caseinolytic activity) either by down regulation of protease expression, secretion or activation, or by up-regulation of a protease inhibitor.

In contrast to the striking effects observed during the first week of culture, short term exposure of MEC cultured in EGF or TGFα medium to PD158780 after day 7 did not affect the profile of casein isoforms expressed at these time points (Figures 6, 8 and 9). Exposure of MEC to PD158780 from day 17.5-21 only altered casein accumulation under conditions when PD158780 was able to induce extensive alveolar regression during this time period (Figure 6 compared with Figure 8 and 9). This selective redistribution in casein isoform

expression may reflect the activity of proteolytic enzymes involved in epithelial apoptosis, selective downregulation of certain casein transcripts, and/or modulation of enzymes involved in the phosphorylation, dephosphorylation or glycosylation of select casein isoforms. The electron photomicrograph in Figure 5F illustrates the dramatic reduction in morphologically distinct milk proteins (likely caseins) within epithelial colonies undergoing alveolar regression as compared to that observed within healthy epithelial colonies (Figure 5E). It should be noted that conditioned medium from these culture wells was not able to degrade purified bovine casein protein when incubated for 2 hours at 37°C (data not shown). Additional studies are required to define the mechanism(s) responsible for the selective redistribution in casein isoforms observed under this specific subset of conditions. It should be emphasized that hrEGF and hrTGFα at 10 ng/ml exhibited equivalent effects of MEC functional differentiation.

B. **AIM 2. BIOLOGICAL ACTIVITY OF EXOGENOUS EGF AND TGFα IN MEC EXPOSED TO THE CARCINOGEN N-METHYL-NITROSOUREA (NMU).**
(Margot Ip, Suzanne Shoemaker, Nannette Stangle, Mary Vaughn, Danilo Zangani, Elizabeth Horn, Collen Tagliarino, Steven Brooks and Ruea Huang carried out various aspects of this work.)

During the last 2 years, considerable effort has been exerted by various members of Dr. Margot Ip's laboratory to establish culture conditions that support the outgrowth of either pre-malignant or malignant MEC isolated from the mammary glands of Sprague-Dawley rats exposed to the carcinogen NMU, or from malignant mammary adenocarcinoma cell lines isolated in the 1970's from mammary adenocarcinomas that developed in Sprague-Dawley rats exposed to either NMU or dimethylbenzanthracene (DMBA) (141). The later cell lines were obtained from the American Type Tissue Culture and are referred to as NMU and RBA cells, respectively.

During this time, our laboratory began using an NMU-induced rat mammary tumor model developed in the laboratory of Dr. Henry Thompson (142). This model system is unique in that it allows rapid induction (5-10 weeks post-carcinogen exposure, rather than 6-9 months) of mammary intraductal hyperplasia, ductal and lobular carcinoma *in situ*, as well as adenocarcinomas following the injection of pre-pubescent female rats with 1-methyl-1-nitrosourea. Initial efforts were exerted on learning how to excise, process and stain mammary gland whole mounts, and then to prepare and embed mammary gland whole mounts for histological characterization and immunohistological analysis. Dr. Thompson and different members of his laboratory were extremely helpful in providing us with detailed protocols and technical advice to set up this model at our facility.

Subsequent efforts were extended towards adapting protocols to isolate MEC from the mammary glands of NMU-exposed rather than normal rats, and in the adaptation of culture conditions that supported the outgrowth of pre-malignant and/or malignant rat mammary tumor cells in monolayer as well as three dimensional serum and serum-free culture conditions. A wide variety of control experiments critical to the establishment of this mammary tumor progression model system are still in progress.

It should be emphasized that the work carried out to establish this model system for mammary tumor progression although fundamental in nature represents a dramatic advancement in the field of breast cancer research. This unique *in vivo* rat mammary tumor

progression model system can now be interfaced with our existing primary culture model. Relevant pre-malignant and malignant MEC, isolated from carcinogen-exposed Sprague-Dawley rats, can now be cultured within the RBM in the presence of defined serum-free culture medium. During the next few months, carcinogen-exposed MEC will be used in primary culture studies originally outlined in this aim to examine the biological activity of exogenous EGF and TGFα, and to determine whether the type and/or the magnitude of the effects induced in pre-malignant and/or malignant MEC are different than the effects observed in the normal MEC. In addition, we hope to determine whether one or both of these polypeptides enhances tumor progression *in vitro* in the absence as well as in the presence of the tumor promoting phorbol esters. Finally, it is hoped that identification of early and/or progressive changes during the progression of breast cancer will identify new targets for the development of effective breast cancer prevention and treatment strategies.

C. AIM 3. SELECTIVE CHANGES IN EGFR EXPRESSION AND INTRACELLULAR LOCALIZATION DURING ALVEOLAR BRANCHING MORPHOGENESIS AND APOPTOSIS OF NORMAL MAMMARY EPITHELIAL CELLS.

(Mary Vaughn, Ann Wohlhueter and Joy Russell assisted in carrying out and analyzing these samples. Ruea Huang, Patricia Masso-Welch and Linda Varela provided many of the *in vivo* developmental samples.)

1. OVERVIEW.

The data collected in aim 1 clearly demonstrated that EGFR activation by EGF or TGFα selectively regulated the proliferation, functional differentiation, and survival of normal rat mammary epithelial cells as well as the level and distribution of casein isoforms. The type and the duration of the effect(s) induced, however, appeared to reflect the state of differentiation of the epithelial cells. During the first week of culture, non-functional MEC underwent extensive proliferation and functional differentiation in response to either EGF or TGFα medium, but not NoGF medium. During the second week of culture, PD158780 exposure did not affect epithelial cell growth, the accumulation of total caseins, or the distribution of casein isoforms expressed by primary MEC. This latter observation suggested that EGFR signaling following activation by EGF or TGFα was no longer involved in regulating these effects. Only MEC cultured for at least 17.5 days in EGF or TGFα underwent extensive apoptosis within 48 hours of being exposed to PD158780.

The results from the primary culture studies led us to speculate that the specific types of effects induced in response to EGF and TGFα during the first week of culture and the last few days of a 21 day study reflected the expression or the intracellular localization of EGFR within the cultured MEC. To test this hypothesis, protocols were established to detect rat EGFR using an Western blot as well as an immunohistochemical procedure. Physiological significance of the *in vitro* profiles was then established by examining epithelial expression (by Western blot analysis) of EGFR in MEC isolated from mammary glands undergoing alveolar branching morphogenesis during pregnancy and lactation as well as undergoing epithelial apoptosis during involution and then by examining intracellular localization (using immunohistochemical analysis) of EGFR within the epithelial and stromal cells of mammary glands during pregnancy, lactation and involution.

EGFR is the most highly studied of the type I tyrosine kinase receptor family (also referred to as the erbB or EGF receptor family). Upon binding ligand, EGFR can homo-dimerize with EGFR, or heterodimerize with erbB2, erbB3 or erbB4. EGF and TGFα specifically bind to EGFR, but not erbB2, erbB3, or erbB4. Signal transduction diversity appears to reflect different internalization rates for the different EGFR dimers. In addition, the cytoplasmic tails of these four receptors are also distinct which allow the different dimers to tyrosine phosphorylate a distinct subset of signal transducers. The specific effects induced in response to EGFR activation by EGF or TGFα in a given cell type are not only affected by the presence of EGFRs on those cells, but also the expression of the other erbB receptors. Western blot studies were therefore undertaken to examine epithelial expression of erbB2, erbB3 and/or erbB4 during different phases of the *in vitro* development of MEC as well as within MEC isolated from mammary glands undergoing alveolar branching morphogenesis and epithelial apoptosis *in vivo*.

EGFR heterodimerization is not only dependent on co-expression of another type of erbB receptor within a given cell type, but also co-localization within a distinct intracellular compartment. An immunohistochemistry protocol was adapted for the detection of rat erbB2, and studies were initiated to examine the intracellular localization of erbB2 within cultured MEC as well as within the various cell types within intact mammary glands during pregnancy, lactation and involution. Immunohistochemistry protocols were recently developed for the detection of rat erbB3 and erbB4, but additional studies are required to examine their distribution during mammary development *in vitro* and *in vivo*.

2.   EXPERIMENTAL METHODS

Whole Cell Lysate Preparation and Western Blot Analysis of EGFR, erbB2, erbB3, erbB4 Expression

EGFR, erbB2, erbB3 and erbB4 expression were examined in whole cell lysates of MEC cultured within the RBM in 100 mm dishes. The RBM within each culture dish was removed prior to lysate preparation. The RBM was either enzymatically digested away using a 30 min 1% (w/v) dispase incubation in culture medium at 37 °C in a gyroshaker set at 200 rpm or mechanically removed by physically disrupting the RBM gel in a high volume of medium, and then carefully removing the RBM which partitioned away from the epithelial organoids following a 10 min centrifugation at ~ 500 x g. Whole cells lysates were then prepared of the isolated cultured MEC using ice-cold 1% (v/v) triton X 100 lysis buffer [50 mM Tris, pH 8.0 with 150 mM NaCl, 2 mM EDTA, 10 mM NaPO$_4$, 10 mM Na pyrophosphate, 5 mM Na orthovanadate, 0.1% (w/v) SDS, 0.5 % (w/v) Na deoxycholate, 0.1 mM PMSF, 100 ng/ml soybean tryspin inhibitor, and 20 ng/ml leupeptin]. Each sample was sonicated on ice for 10 sec each using a Tekmar sonic disruptor, vortexed for 10 min at 4°C, and microfuged at ~12,000 x g at 4°C. Supernatants were aliquoted, quick frozen in liquid nitrogen and stored at -20°C.

EGFR, erbB2, erbB3 and erbB4 expression were also examined in MEC isolated from the mammary glands of pubescent virgin female, mid-pregnant, lactating or involuting rats. Abdominal and inguinal mammary glands excised from (1) 50- to 54-day-old, (2) mid-pregnant (day 14-16), (3) lactating (day 5, 10, 15 or 21), or involuting (day 3 or day 7) Sprague-Dawley rats were mechanically and enzymatically disaggregated. The pooled

mammary glands were minced into small fragments and then cultured at 37°C in digestion medium [10 ml/g] (0.2% [w/v] collagenase type III, 0.2% [w/v] dispase grade II, 5% newborn calf serum and 50 µg/ml gentamycin in PRF RPMI 1640). Mammary glands from virgin, mid-pregnant, lactating and involuting rats were digested for ~14.5, 4.5, 4.5, and 5 hours, respectively. MEC were isolated from the digests as described above for the isolation of MEC for the primary culture studies. Trizol samples were prepared as recently described by Varela and Ip (143) from the non-adherent organoids from each of these samples and proteins within the Trizol samples solubilized in 10 M urea and 50 mM DTT.

Appropriate supernatant lysates or Trizol protein samples were mixed with Laemmli reducing and denaturing sample buffer, boiled, and separated on 4-20% gradient polyacrylamide gels. Proteins within the gels were transferred to Immobilon P membranes. EGFR Western blots were carried out on the membranes using 1 µg/ml of either an affinity-purified polyclonal sheep anti-peptide antibody against a portion of the cytoplasmic tail of the human EGFR (UBI 06-129) or a sheep IgG whole molecule (isotype control). Immunoreactive proteins were detected on X-ray film using a horseradish peroxidase conjugated donkey anti-sheep secondary antibody and an enhanced chemiluminescence (ECL) reagent. ErbB2, erbB3 and erbB4 were detected using 1 µg/ml of either a specific affinity-purified polyclonal rabbit anti-peptide antibody raised against sequences within the cytoplasmic tail of erbB2 (SC-284), erbB3 (SC-285) or erbB4 (SC-283), or a rabbit Ig whole molecule. These immunoreactive proteins were detected on X-ray film using a horseradish peroxidase conjugated donkey anti-rabbit secondary antibody and an ECL reagent. X-ray films were digitally scanned using an AGFA flatbed scanner, images processed using Adobe Photoshop and Powerpoint, and images printed using a Hewlett Packard LaserJet 5L printer. Peptide competition Westerns were also performed for the different Santa Cruz anti-peptide antibodies. Ten µg of either SC-284, SC-285 or SC-283 immunizing peptide was pre-incubated with 1 µg of the respective SC-284, SC-285, or SC-283 anti-peptide antibodies overnight at 4°C. Western blot assays were then performed using the different affinity-purified antibody pre-mixed with the respective immunizing peptide.

Immunohistochemical Detection of Rat EGFR and ErbB2

Intracellular localization of EGFR and erbB2 was detected within formalin-fixed and paraffin-embedded MEC cultured within the RBM in NoGF, EGF or TGFα medium for 2 hours, 3.5 days, 7 days, 14 days or 21 days. Alternatively, EGFR and erbB2 levels were examined in the epithelial and stromal cells of intact formalin-fixed and paraffin-embedded abdominal and occasionally inguinal mammary glands excised from (1) pubescent virgin female (50- to 54-day-old), (2) pregnant (day 7 or day 14-16), (3) lactating (day 5-7, 15 or 21), or involuting (day 3-4, day 6-7 or day 9-10) Sprague-Dawley rats.

Paraffin-embedded blocks of cultured MEC as well as intact mammary glands were sectioned (4-5 µm), collected onto 3-aminopropyltriethoxylsilene-treated slides, de-paraffinized, quenched for endogenous peroxidase activity, blocked with 0.03% (w/v) casein in PBS. Tissue sections were incubated sequentially with (1) 1µg/ml of an affinity-purified polyclonal rabbit anti-peptide antibody raised against a specific sequence of the cytoplasmic tail of either EGFR (SC-03) or ErbB2 (SC-284), (2) a pre-absorbed donkey anti-rabbit antibody conjugated with biotin, and (3) a streptavidin-horseradish peroxidase conjugate. Immunoreactive proteins were visualized with the brown chromogen DAB. Sections were

counterstained for 1 minute with hematoxylin, dehydrated and coverslips mounted using Permount.

Mammary glands from at least three different rats were evaluated at each of the time points given above. During involution, abdominal mammary glands were subdivided into three pieces based on proximity of the piece to the abdominal mammary gland nipple. At least one animal for each of the major developmental stages was selected and in that rat bilateral abdominal glands were analyzed. All tissue sections were evaluated in at least two independent immunohistochemistry assays for EGFR as well as erbB2.

Each tissue section was evaluated with [1] **SC-03 alone** (1 μg/ml of SC-03 antibody pre-mixed overnight at 4°C with PBS), [2] **SC-03 competition with SC-03P** (1 μg/ml of SC-03 antibody pre-mixed overnight at 4°C with 10 μg of the immunizing SC-O3 peptide), [3] **SC-284 alone** (1 μg/ml of SC-284 antibody pre-mixed overnight at 4°C with PBS), as well as [4] **SC-284 competition with SC-284P** (1 μg/ml of SC-284 antibody pre-mixed overnight at 4°C with 10 μg of the immunizing SC-284 peptide).

Random tissue sections from each developmental stage were then evaluated with [1] **isotype control** (1 μg/ml rabbit IgG whole molecule), [2] **SC-03 competition with a non-specific peptide** (1 μg/ml of SC-03 antibody pre-mixed overnight at 4°C with 10 μg of the SC-284 peptide), as well as [3] **SC-284 competition with a non-specific peptide** (1 μg/ml of SC-284 antibody pre-mixed overnight at 4°C with 10 μg of the SC-O3 peptide).

Immunohistochemical results were independently evaluated at 400 X magnification using either an Olympus BH-2 microscope or an Olympus BX-40 microscopic by at least three individuals. Photographs were taken using an Olympus BH-2 microscope mounted with a Nikon FX-35A camera using either Fuji or Kodak 200 ASA color print film.

3.   RESULTS and DISCUSSION.

EGFR LEVELS SELECTIVELY CHANGED AS THE CULTURED MEC UNDERWENT BRANCHING ALVEOLAR MORPHOGENESIS AND THEN APOPTOSIS *IN VITRO*

Studies were carried out to test the hypothesis that the type of effect induced in response to EGFR activation by EGF or TGFα reflected the level of EGFR expressed on the MEC. Initial studies were undertaken to evaluate the level of EGFRs expressed in primary MEC cultured under defined serum-free conditions that supported branching alveolar morphogenesis. Functionally immature MEC cultured in EGF or TGFα medium were previously shown to proliferate, morphologically and functionally differentiate, and undergo branching alveolar morphogenesis during the course of a 21 day study in a manner reminiscent of that of luminal epithelial cells within intact mammary glands during pregnancy and lactation (74,105-107). In contrast, MEC cultured in NoGF medium without an exogenous EGFR ligand underwent modest proliferation, differentiation and branching morphogenesis.

**EGFR Expression was Inversely Related to the
Degree of Functional Differentiation of the Cultured MEC**

Levels of EGFR were evaluated in the cultured MEC by Western blot analysis using an affinity-purified polyclonal sheep anti-recombinant protein antibody corresponding to a portion of the cytoplasmic domain of the human EGFR (UBI 06-129). High levels of EGFR were observed in the functionally undifferentiated MEC isolated from pubescent female virgin rats at 2 hrs in culture (Figures 10-12). In the presence of NoGF, EGF or TGFα medium, EGFR levels varied during the course of the 21 day study with a decrease observed during the first week, an increase by day 10.5, and then a progressive decrease during the remainder of the study (Figures 10-12). Extremely low levels of EGFR were observed during the third week of culture in EGF or TGFα medium when most of the MEC had morphologically and functionally differentiated into multilobular alveolar organoids (Figures 11 and 12, respectively). During the course of the 21 day study, EGFR expression in MEC cultured in NoGF medium (Figure 13) was not down-regulated to the same extent as that observed when MEC cultured in either EGF or TGFα medium (Figures 11 and 12). MEC cultured in NoGF medium accumulated less casein than MEC cultured in EGF medium (Figure 3B and 4B).

High levels of EGFR were observed within the newly isolated immature and cytologically differentiated MEC, moderate levels of EGFR were observed in the cultured MEC as they became functionally differentiated, and low levels of EGFR were observed in the fully functional MEC. This data seemed to suggest that EGFR expression is inversely related to the degree of functional differentiation of MEC. This hypothesis is supported by binding studies using ^{125}I-EGF that demonstrated the presence of relatively high numbers of binding sites for EGF in membrane preparations of mammary glands from virgin mice (144). The level of binding sites for EGF within mammary gland membrane preparations were then observed to decrease during early pregnancy, to increase during mid-pregnancy, progressively decrease during late-pregnancy and then to remain low throughout lactation (144). Furthermore, binding studies using ^{125}I-TGFα demonstrated that microsome fractions prepared from the mammary glands of non-pregnant and mid-pregnant sheep contained 3-fold more binding sites than in mammary glands from sheep during late-pregnancy and lactation (145). Long term exposure of MEC to EGF or TGFα probably played a role in down-regulating epithelial expression of EGFR. EGF at 10 ng/ml was shown to down-regulate the number of ^{125}I-EGF binding sites in primary mouse MEC cultured in serum-free collagen-gel culture (146).

These studies also demonstrated that EGFRs were expressed with a mass of 164-170 kDa and/or 150 kDa in the cultured MEC. The 150 kDa form may reflect the proteolytic degradation of native EGFR during the dispase digestion required to remove the large quantities of proteins present within the RBM in these samples. The 150 kDa form of EGFR was described in membrane preparations of rat liver and hepatoma tissue (147) as well as within primary mouse MEC cultured in a collagen-gel system (148). Both papers suggested that the 150 kDa form represents a proteolytic fragment of the full length 170 kDa EGFR.

Functionally Immature MEC Expressed High Levels of Surface EGFR

Intracellular localization of EGFRs within the cultured MEC was analyzed in formalin-fixed and paraffin-embedded MEC cultured for 0, 3.5, 7, 14 or 21 days within the RBM in EGF medium using an affinity-purified rabbit polyclonal anti-peptide antibody raised against a fragment of the cytoplasmic tail of the EGFR (SC-03 at 1 μg/ml) and visualized with the

brown chromogen DAB. Figure 13A illustrates that MEC cultured for 2 hours (day 0) expressed moderate to high levels of cytoplasmic as well as plasma membrane-associated EGFR. EGFRs were uniformly expressed in many of the epithelial cells within the individual colonies at day 0. Relative to the day 0 MEC, many of the MEC within the developing lobular, multilobular as well as lobuloductal colonies expressed moderate levels of plasma membrane and cytoplasmic EGFR by day 3.5 and day 7 of culture (Figures 13B and 13C, respectively).

Fully Functional MEC Expressed Low Levels of Surface EGFR

By day 14 and day 21 of culture in EGF medium, most MEC were organized into extremely large and well developed multilobular alveolar organoids. These luminal epithelial cells were functionally differentiated with morphological evidence of the accumulation of extensive intracellular lipid and modest to abundant quantities of secreted protein (likely casein) within distended internal lumen. MEC that developed *in vitro* for 14 or 21 days in EGF medium expressed moderate to low levels of EGFR that were predominantly localized around intracellular lipid (Figures 13D and 13E, respectively). Additional studies are required to determine whether EGFR localized around intracellular lipid play a functional role or simply reflects residual evidence of internalized EGFR.

In summary, the immunohistochemical results demonstrated that functionally immature MEC expressed the highest levels of plasma membrane-associated EGFR, and that plasma membrane-associated EGFR progressively decreased as MEC underwent branching alveolar morphogenesis *in vitro*. Functionally differentiated MEC that produced large quantities of milk lipid and casein, expressed the lowest levels of plasma membrane-associated EGFR. In addition, this data confirmed the Western blot data that suggested that EGFR expression was inversely related to the degree of functional differentiation of the cultured MEC.

Cultured MEC Undergoing Apoptosis Re-Expressed
High Levels of Membrane and Cytosolic EGFR

Studies were then carried out to evaluate EGFR levels within terminally differentiated MEC that underwent apoptosis *in vitro* in a manner reminiscent of that observed within intact mammary glands during involution. As indicated in aim 1, MEC cultured in either EGF or TGFα medium for at least 17 days underwent extensive apoptosis when the cells were then exposed to 0.5 μM PD158780 for a 48 hour time period. EGFR expression and intracellular localization were examined using an immunohistochemistry assay on serial sections of the same formalin-fixed, paraffin-embedded epithelial organoids used for the *in situ* apoptotsis detection assay (TUNEL assay) presented in Aim 1. EGFRs were detected with the rabbit polyclonal anti-peptide antibody raised against a fragment of the cytoplasmic tail of the EGFR (SC-03 at 1 μg/ml). Interestingly, the epithelial organoids that naturally underwent apoptosis *in vitro* re-expressed high levels of immunoreactive EGFR visualized with the brown chromogen DAB (Figure 5I).

Specificity of the brown EGFR staining (Figure 5I) was examined by antibody competition using the immunizing EGFR peptide (SC03P) (Figure 5J) or a peptide corresponding to a specific fragment of the cytoplasmic tail of ErbB2 (SC284P) (data not

shown). The EGFR peptide (SC03P), but not the ErbB2 peptide (SC284P) was able to compete for the affinity-purified anti-EGFR peptide antibody (SC-03) and thereby eliminate all of the brown EGFR staining observed within epithelial colonies undergoing alveolar regression (apoptosis). An isotype control group was also analyzed using rabbit IgG whole molecule (JIRL 011) and none of the cultured MEC stained brown with the rabbit IgG whole molecule at 1 µg/ml (data not shown).

MEC Undergoing Apoptosis *In Vitro* Expressed High
Levels of Membrane and Cytoplasmic ErbB2

Interestingly, the epithelial organoids that naturally underwent apoptosis *in vitro* also expressed high levels of immunoreactive erbB2 visualized with the brown chromogen DAB (Figure 5K). Specificity of the brown erbB2 staining (Figure 5K) was confirmed by antibody competition analysis using the immunizing erbB2 peptide (SC-284P) (Figure 5L).

WESTERN BLOT DETECTION OF MAMMARY EPITHELIAL EGFR
EXPRESSION DURING BRANCHING ALVEOLAR
MORPHOGENESIS AND EPITHELIAL APOPTOSIS *IN VIVO*

To evaluate the physiological significance of the EGFR expression profile observed during the *in vitro* development of MEC, EGFR expression was evaluated by Western blot analysis in MEC isolated from the mammary glands of 50-54 day old pubescent female virgin rats, and rats during mid-pregnancy (day 14-16), lactation (day 5, 7, 10, 15 or 21), or involution (day 3 and/or day 7). EGFR levels were evaluated using a polyclonal sheep anti-fusion protein antibody raised against a portion of the cytoplasmic tail of the human EGFR (UBI 06-129).

In order to selectively examine mammary epithelial expression by Western blot analysis of mammary glands during different stages of development, it was first necessary to separate the MEC from the surrounding stromal tissue containing variable amounts of adipocytes, fibroblasts, immune cells, and vessels involved in the circulatory, lymphatic as well as nervous systems. Protein lysates were prepared of isolated MEC rather than from the entire mammary gland because the proportion of epithelial cells and stromal cells within intact mammary glands changes dramatically during the different developmental stages. For example, mammary glands from pubescent virgin female rats contain high numbers of mature adipocytes and relatively low numbers of MEC whereas mammary glands from lactating female rats have high numbers of MEC and low numbers of mature adipocytes.

Epithelial expression of EGFR was high within the mammary glands of virgin rats, progressively decreased during pregnancy and lactation, and then increased during involution (Figure 14). Two immunoreactive bands were observed in the isolated MEC. The slower migrating form was 165-170 kDa whereas the faster migrating form was ~ 150 kDa in size. The 150 kDa protein may reflect the proteolytic degradation of full length EGFR as a result of exposure of the MEC to collagenase and dispase during the MEC isolation procedure. An 150 kDa proteolytic fragment of the EGFR was described in membrane preparations of rat liver and hepatoma tissue (147) as well as primary mouse MEC cultured in a collagen-gel system (148).

IMMUNOHISTOCHEMICAL LOCALIZATION OF EGFR WITHIN MAMMARY STROMAL AND EPITHELIAL CELLS DURING BRANCHING ALVEOLAR MORPHOGENESIS AND EPITHELIAL APOPTOSIS *IN VIVO*

EGFR levels and immunolocalization were also examined within epithelial and stromal cells within intact formalin-fixed and paraffin-embedded mammary glands excised from rats during similar developmental stages as those used in the western blot studies. Mammary gland sections stained with 1 µg/ml rabbit IgG were devoid of brown staining in mammary epithelium, fibroblasts and adipocytes (data not shown). Overnight preincubation of 1 µg/ml SC-03 antibody with 10 µg/ml of the immunizing SC-03 peptide completely eliminated the brown staining observed in mammary epithelial cells, fibroblasts as well as adipocytes (data not shown). Serial sections of all samples were evaluated using the affinity-purified SC-03 antibody alone or the SC-03 antibody preincubated with the immunizing SC-03 peptide.

Luminal Epithelial Cells within Mammary Glands of Virgin Rats Expressed Apical, Basal and/or Lateral EGFRs

Luminal MEC within mammary gland alveolar buds (Figure 15 panels A1, and A5), terminal end buds (Figure 15 panel A2) as well as ducts (Figure 15 panels A3, A6 and A7) from virgin rats exhibited distinct membrane as well as cytoplasmic EGFR. When examining the staining within the luminal epithelium of alveolar buds, these well polarized epithelial cells exhibited concentrated and intense basal, lateral and/or apical membrane staining along with modest cytoplasmic staining (Figure 15 panels A1, and A5-A6).

Within terminal end bud structures, the cells lining the outer surface of the structure, the "cap cell layer", appeared to stain more intensely than the more luminal non-polarized epithelial cells, and were positioned adjacent to intensely stained stromal fibroblasts. The more luminal epithelial cells within the terminal end buds were variably stained with certain cells exhibiting distinct plasma membrane staining around the entire plasma membrane, others showing diffuse cytoplasmic staining and finally some that were clearly unstained (Figure 15 panel A2).

When examining the staining within the ductal luminal epithelial cells two general patterns were observed. The first pattern was marked by distinct and intense apical membrane staining as well as diffuse cytoplasmic staining along a majority of the cells lining certain ducts (Figure 15 panel A6). The second pattern consisted of well polarized epithelial cells that lacked apical membrane staining, but contained intense concentrated basal and/or lateral membrane staining (Figure 15 panels A3 and A7, respectively).

Intense plasma membrane-associated EGFR staining was observed within all mammary fibroblasts and adipocytes observed throughout the mammary gland of pubescent virgin rats regardless of proximity to mammary epithelial cells (Figure 15 panels A3, A4, and A6-A8).

Luminal MEC Expressed Perinuclear as well as Recticular-Cytoplasmic EGFR during Pregnancy

By mid-pregnancy, high proportions of well polarized luminal epithelial cells were organized in alveolar clusters around small central lumen. Epithelial staining within alveolar clusters ranged from moderate intensity primarily localized as diffuse cytoplasmic and perinuclear EGFR (Figure 15 panel B1) to high intensity localized as reticular-cytoplasmic and apical membrane EGFR (Figure 15 panel B2). Epithelial staining within ducts also varied from intense apical, basal and/or lateral EGFR staining (Figure 15 panel B3) to modest staining primarily localized as diffuse perinuclear. The moderate intensity perinuclear EGFR staining was observed within ~ 30-70% of luminal epithelial cells within alveolar clusters.

During pregnancy, EGFR staining in mammary fibroblasts was moderate in intense (Figure 15 panel B3), whereas adipocyte staining was variable and ranged from moderate to a very light intensity (Figure 15 panel B4). The intensity of adipocyte staining appeared to directly corresponded to the intensity of reticular-cytoplasmic staining observed in luminal epithelial cells within alveolar clusters.

Additional studies are required to determine the biological significance of perinuclear and/or reticular-cytoplasmic EGFR localization within luminal MEC. Different types of cytoplasmic EGFR may simply reflect distinct stages during the internalization and degradation of EGFR. Alternatively, variant EGFRs might be expressed that fail to insert themselves into the plasma membrane, are preferentially localized in a perinuclear and/or reticular localization, and tyrosine phosphorylate a select subset of (SH2-containing) proteins involved in regulating cell shape, protein synthesis, post-translational modifications and/or vectoral secretion.

Fully Functional MEC Expressed Low Levels of
Plasma Membrane-Associated EGFR during Lactation

During lactation, most luminal mammary epithelial cells exhibit a cuboidal shape and are organized in clusters of alveoli with distended central lumen. EGFR staining within many of these cells was very low in intensity (Figure 15 panel C2). Certain luminal epithelial cells exhibited a distinct moderate intensity perinuclear localization (Figure 15 panel C1). Modest apical membrane staining was apparent within some luminal epithelial cells and very limited basal membrane staining was observed (Figure 15 panel C1). In contrast, myoepithelial cells surrounding the individual alveoli were devoid of stained EGFR (Figure 15 panel C2). It should be noted that staining intensity among alveoli was not affected by the dimensions of the central lumen or the apparent functional status of the alveolar cluster in question. Luminal epithelial cells lining ducts exhibited a generalized diffuse EGFR staining profile that was moderate to dark in intensity (Figure 15 panel C3).

Although the stromal compartment of the mammary gland during lactation is extremely small, modest to low intensity EGFR staining was observed within mammary fibroblasts (Figure 15 panel C3) and adipocytes (Figure 15 panel C4) during the first or second week of lactation.

Luminal MEC Undergoing Apoptosis during Involution
Re-Expressed High Levels of Membrane and Cytoplasmic EGFR

By day 21 of lactation (also referred to a 12 hours of involution since the 21 day-old pups were removed 12 hours before excision and formalin-fixation of the abdominal mammary glands), luminal epithelial cells within alveolar clusters exhibited moderate to dark cytoplasmic as well as apical EGFR staining when the central lumen was devoid of morphological evidence of secreted milk products (Figure 15 panel C5), whereas alveolar clusters surrounding lumen with morphologic evidence of secreted milk products exhibited a low to modest degree of EGFR staining (Figure 15 panel C6). Apical EGFR staining was observed in many of the luminal epithelial cells that lined mammary gland ducts during this transition from lactation to involution. Mammary gland stromal cells expressed moderate intensity EGFR staining at this stage (Figure 15 panels C7 and C8).

Sprague-Dawley rat mammary glands appeared to exhibit the most extensive morphologic signs of apoptosis of alveolar luminal epithelial cells by day 3-4 of involution. During this stage of involution, mammary fibroblasts as well as adipocytes stained intensely for EGFR (Figure 15 panel D3 and D4). Dark EGFR staining was also observed within the cytoplasm and along the plasma membranes of all of the luminal epithelial cells within alveolar clusters and ducts of mammary glands from rats at day 3-4 of involution (Figure 15 panels D1-D3). Morphologically distinct myoepithelial cells were primarily devoid of EGFR staining. By day 6-10 of involution, EGFR staining within the stromal and luminal epithelial cells decreased to a moderate to low intensity range (Figure 15 panel D5-D8).

These *in vivo* findings demonstrate that high levels of plasma membrane-associated EGFR were expressed in many immature and cytologically differentiated epithelial cells of mammary glands from virgin female rats as well as in all ductal and alveolar luminal MEC of mammary glands during day 3-4 of lactation, but not in fully functionally differentiated MEC during lactation. The observation that all luminal epithelial cells within day 3-4 rat mammary glands expressed high levels of membrane and cytoplasmic EGFR even though not all of these cells will undergo apoptosis suggests that regulation of mammary epithelial apoptosis is a complex process, not solely dependent on EGFR signaling.

Taken together, the *in vivo* immunohistochemical and Western blot findings confirm that the EGFR profiles observed within the cultured MEC were physiologically relevant. This primary culture model system is, therefore, ideal to study regulators of EGFR expression, intracellular localization as well as mammary epithelial apoptosis.

PRIMARY MEC SELECTIVELY EXPRESSED EGFR, ERBB2, ERBB3 AND ERBB4 DURING BRANCHING ALVEOLAR MORPHOGENESIS AND APOPTOSIS *IN VITRO*

Western blot studies were then carried out to determine whether cultured MEC co-expressed EGFR, erbB2, erbB3 and erbB4. Thus far analysis have only been carried in lysates from MEC cultured for up to 21 days in EGF medium. Newly isolated MEC expressed peak levels of EGFR (Figures 16A and 16B). As indicated above, EGFR dramatically decreased during the third week of culture (Figures 16A and 16B) when a majority of MEC were functionally differentiated and accumulating large quantities of milk protein and lipid (Figures 4B and 5D). MEC undergoing apoptosis *in vitro* were also shown to express high levels of EGFR and erbB2 (Figure 5 panels I and K). Cultured MEC expressed peak levels of erbB2 (Figure 16C) and erbB3 (Figure 16D) from day 7-14 of culture whereas erbB4 levels were only detected within MEC cultured for 7 and 10.5 days (Figure 16E).

This data may suggest that during the first week of culture in EGF medium that EGFR homodimers play a dominant role in mediating the mitogenic and lactogenic effects of EGF whereas EGFR heterodimers with erbB2, erbB3 or erbB4 play more important roles in transducing EGF-dependent effects during the second week of culture. Additional studies are required, however, to identify the biological effect(s) induced by EGF during the second week of culture. The finding that cultured MEC undergoing apoptosis expressed high levels of EGFR and erbB2 may support the hypothesis that signaling through EGFR-erbB2 heterodimers plays a significant role in dictating whether or not terminally differentiated MEC undergo apoptosis.

MEC UNDERGOING BRANCHING ALVEOLAR MORPHOGENESIS AND APOPTOSIS *IN VIVO* EXPRESSED DISTINCT LEVELS OF EGFR, ERBB2, ERBB3 AND ERBB4

Western blot analysis was then carried out to determine whether MEC isolated from mammary glands of normal rats during the various developmental stages expressed the different erbB receptors, and then to evaluate whether or not these receptors were coordinately regulated. High levels of EGFR, erbB2 and erbB3 were expressed in MEC isolated from virgin rats as well as rats during pregnancy and involution, and lower levels were observed in MEC during lactation (Figures 17A-17C). In contrast, erbB4 appeared to be uniformly expressed throughout the developmental stages analyzed.

Although this data suggests that EGFR, erbB2 and erbB3 may, in part, be coordinately regulated during the development of normal MEC *in vivo*, the procedure to isolate MEC utilized a collagenase and dispase digestion step which likely cleaved plasma membrane-associated erbB receptors into the multiple forms observed in Figure 17. It also seems reasonable that dissociation of MEC from their surround ECM and stroma might regulate erbB expression. Immunohistochemical protocols were, therefore, developed to examine the natural expression and intracellular localization of the different erbB receptors. Thus far, evaluation of have only been carried out for EGFR (Figure 15) and erbB2 (see below).

INTRACELLULAR LOCALIZATION OF ERBB2 WITHIN CULTURED MEC

Intracellular localization of erbB2 within the cultured MEC was analyzed in formalin-fixed and paraffin-embedded MEC cultured for 0, 3.5, 7, 14 or 21 days within the RBM in EGF medium using a rabbit polyclonal anti-peptide antibody raised against a fragment of the cytoplasmic tail of the erbB2 receptor (SC-284 at 1 µg/ml) and visualized with the brown chromogen DAB. Figure 18A illustrates that MEC cultured for 2 hours (day 0) expressed low levels of erbB2 receptor. Relative to the day 0 MEC, many of the MEC within the developing lobular, multilobular as well as lobuloductal colonies expressed moderate to high levels of plasma membrane and cytoplasmic erbB2 by day 3.5 and day 7 of culture. Specifically, erbB2 was expressed in distinct regions of the basal plasma membrane (Figures 18B and 18C).

By day 14 and day 21 of the study, most MEC cultured in EGF medium were organized into large multilobular alveolar organoids, and were functionally differentiated with morphological evidence of the accumulation of extensive intracellular lipid and modest to

abundant quantities of secreted protein (likely casein) within distended internal lumen. MEC that developed *in vitro* for 14 or 21 days in EGF medium expressed moderate to low levels of cytoplasmic erbB2, and retained the distinct basal localization seen at earlier times in culture as well as sharp staining around intracellular lipid (Figures 18D and 18E) These results suggest that newly isolated functionally immature MEC expressed the lowest levels of erbB2, and that plasma membrane-associated erbB2 expression peaked at day 7 as the MEC underwent branching alveolar morphogenesis *in vitro*. ErbB2 expression appeared to decrease as the MEC accumulated increasing quantities of milk lipid and protein and to localize around intracellular lipid. The biological significance of the localization of erbB2 around intracellular lipid remains to be determined.

IMMUNOHISTOCHEMICAL LOCALIZATION OF ERBB2 WITHIN MAMMARY STROMAL AND EPITHELIAL CELLS DURING BRANCHING ALVEOLAR MORPHOGENESIS *IN VIVO*

Immunohistochemical studies were then carried out to evaluate potential physiological relevance of EGFR-erbB2 heterodimers in mediating specific events during branching alveolar morphogenesis *in vivo* by defining situations when these two receptor were co-expressed in the same intracellular localization. Intracellular localization of erbB2 receptors was, therefore, examined in serial sections of the same developmental paraffin blocks used in EGFR immunohistochemistry studies. ErbB2 expression was visualized with the brown chromogen DAB. Sections stained with 1 µg/ml rabbit IgG were devoid of brown staining in mammary epithelium, fibroblasts and adipocytes. Overnight preincubation of 1 µg/ml SC-284 antibody with 10 µg/ml of the immunizing SC-284 peptide completely eliminated the brown staining observed in mammary epithelial cells, fibroblasts as well as adipocytes. Serial sections of all samples were evaluated using the SC-284 antibody alone or with the SC-284 antibody preincubated with the immunizing SC-284 peptide.

Mammary Gland Expression of ErbB2 in Virgin Rats

In general, ErbB2 was expressed at high levels within the mammary glands of 50-54 day-old virgin female rats. Individual luminal epithelial cells within alveolar bud and ducts, however, varied in erbB2 staining intensity and localization. Many alveolar luminal MEC exhibited intense apical membrane erbB2, reticular cytoplasmic erbB2, and general erbB2 staining along all membranes including those surrounding lipid droplets (Figure 19 panels A1, A2 and A6). In addition, alveolar MEC displayed a low level of erbB2 diffusely localized throughout the cytoplasm, or did not contain any erbB2 staining (Figure 19 panel A5). Luminal MEC within certain ducts exhibited high levels of diffuse and reticular erbB2 in their cytoplasm (Figure 19 panel A3), whereas those in other ducts displayed low levels of diffuse cytoplasmic erbB2 (Figure 19 panel A7). Moderate reticular cytoplasmic and intracellular membrane staining was observed in terminal end buds (Figure 19 panel A2).

Adipocytes exhibited dark to moderate diffuse ErbB2 staining along their plasma membranes in regions proximal as well as distal to the mammary epithelial cells (Figure 19 panels A4 and A8). Fibroblasts on the other hand showed more intense staining when in close proximity to mammary epithelial cells (Figure 19 panels A3, and A5-A7). Fibroblasts exhibited distinct regions of reticular ErbB2 staining possibly related to the association of erbB2 with cytoskeletal proteins.

ErbB2 Expression in Mammary Glands of Pregnant Rats

ErbB2 was expressed at relatively low levels in mammary glands from pregnant compared with virgin rats. Alveolar clusters exhibited two basic staining patterns for ErbB2 in mammary gland from mid-pregnant rats. Reticular cytoplasmic staining and membrane staining around lipid droplets were observed in luminal MEC organized in well polarized alveolar clusters with central lumen (Figure 19 panel B1). In contrast, alveolar clusters that lacked a central lumen and were composed of less polarized cells preferentially displayed distinct perinuclear and diffuse cytoplasmic erbB2 (Figure 19 panel B2). Ductal MEC were shown to possess diffuse cytoplasmic and lateral membrane erbB2 as well as erbB2 localized along intracellular membranes (Figure 19 panel B3). A few ductal epithelial cells exhibited perinuclear erbB2 in addition to cytoplasmic and membrane erbB2.

Low level plasma membrane staining was observed in adipocytes throughout mammary glands during pregnancy (Figure 19 panel B4). In addition, most fibroblasts expressed low levels of erbB2 or no erbB2 staining (Figure 19 panel B3), and a small percentage displayed punctate erbB2.

ErbB2 Expression in Mammary Glands of Lactating Rats

ErbB2 was expressed at moderate levels in epithelium and low levels in stroma throughout lactation and into involution. In early (day 7) to mid (day 15) lactation two basic staining patterns were observed in alveolar epithelial cells. In early lactation, alveolar epithelial cells expressed diffuse cytoplasmic and intracellular membrane erbB2 (Figure 19 panel C1). Similar to that seen during pregnancy, distinct perinuclear erbB2 was observed in alveolar epithelial cells during mid-lactation (Figure 19 panel C2).

Late in lactation/early involution, alveolar epithelial cells expressed erbB2 along their plasma membrane as well as intracellular membranes. Low levels of erbB2 were observed in alveolar clusters that contained erbB2 positive luminal products (Figure 19 panel C6). Luminal erbB2 likely arose during the process of milk lipid secretion in these alveoli. Membrane bound lipid is vectorally secreted into alveolar lumen and the milk fat globule derives its membrane from portions of the apical plasma membrane. In contrast, alveolar clusters that lacked luminal products primarily expressed erbB2 along the apical plasma membranes of these luminal MEC (Figure 19 panel C5). Ductal epithelial cells exhibited intense apical plasma membrane erbB2 and in most cases were devoid of any other staining (Figure 19 panel C7), although early in lactation some cytoplasmic staining was also observed in the ductal structures (Figure 19 panel C3).

Virtually no ErbB2 staining was observed in adipocytes at any time during lactation except in a few cases where a very light cytoplasmic stain was observed (Figure 19 panels C4 and C8). Similarly, low levels of staining were observed in fibroblasts during early lactation- day 5 (Figure 19 panel C3), but at day 21 lactation/12 hour involution a light, diffuse cytoplasmic stain was observed (Figure 19 panel C7).

Mammary Gland Expression of ErbB2 During Involution

ErbB2 expression during involution was variable depending on proximity to the nipple and time during involution. By day 3-4 of involution, intense reticular cytoplasmic and plasma membrane erbB2 was observed within luminal MEC within alveolar clusters (Figure 19 panels D1, D2 and D5) and ducts (Figure 19 panel B3) in a region of the mammary gland that is furthest from the nipple), whereas low levels of diffuse erbB2 were observed within luminal epithelial cells of alveolar clusters (Figure 19 panel D6) or ducts (Figure 19 panel D7) in the region of the mammary gland closest to the nipple (Figure 19 panel D6). By day 6-10 involution, most alveolar and ductal luminal MEC exhibited diffuse cytoplasmic erbB2 that was light to moderate in intensity (Figure 19 panels D9-D11).

At day 3-4 involution, adipocytes farthest away from the nipple exhibited moderate levels of plasma membrane erbB2 in the regions of the mammary gland that were furthest from the nipple (Figure 19 panel D4), and very low levels of plasma membrane erbB2 in regions of the mammary gland that were closest to the nipple (Figure 19 panel D8). By day 6-10 involution, adipocytes uniformly exhibited a modest level of erbB2 throughout the gland (Figure 19 panel D12). Fibroblasts were even more variable at day 3-4 involution. Moderate levels of cytoplasmic erbB2 were observed in fibroblasts that were furthest away from the nipple (Figure 19 panel D3), whereas fibroblasts that were closest to the nipple were often negative for erbB2 (Figure 19 panel D7). An intermediate level of cytoplasmic erbB2 was observed in fibroblasts by day 6-10 involution (Figure 19 panel D11). During this time, certain fibroblasts also exhibited perinuclear staining.

In summary, ErbB2 expression was highest in virgin mammary gland and decreased during pregnancy. During lactation, ErbB2 levels were moderate in the epithelium and low in the stroma. ErbB2 levels peaked at day 3-4 involution in regions of the mammary gland most distal from the nipple, and by day 6-10 involution erbB2 levels decreased to levels below that observed within the mammary glands of virgin rats. ErbB2 levels and intracellular localization changed dramatically during the different stages of development and as such erbB2 expression is likely under the control of ovarian and pituitary homrones.

EGFR and erbB2 were often localized within the same intracellular compartments of luminal MEC during distinct phases of branching alveolar morphogenesis and apoptosis *in vivo* which supports the hypothesis that EGFR-erbB2 heterodimers play significant roles in regulating epithelial cell shape, migration, invasion and/or survival. Additional studies are required, however, to demonstrate that co-localized erbB receptors are functionally active during the different stages of mammary gland development and then to define the distinct signal transductions pathways induced in response to EGFR-erbB2 heterodimer as compared to EGFR homodimer activation. Taken together, these studies demonstrate a dramatic advance for those studying erbB receptor biology during normal development. It is hoped that these findings will provide the foundation whereby the role(s) that erbB receptors play in regulating the development and progression of breast cancer cells can be more accurately interpreted.

D.   **AIM 4. COMPARATIVE ANALYSIS OF THE DISTRIBUTION OF EGFR WITHIN NORMAL AND VARIOUS TYPES OF TRANSFORMED MEC CULTURED WITHIN A COMPLEX RBM IN THE PRESENCE OF DEFINED SERUM-FREE CULTURE MEDIUM.**

These studies were designed to examine the expression and intracellular localization of EGFR within pre-malignant as well as malignant lesions within mammary glands of rats exposed to the carcinogen N-methylnitrosourea or dimethylbenzanthracene. We were also interested in examining stromal expression of EGFR within and surrounding these various types of lesions within carcinogen-exposed mammary glands. Thus far, approximately 50% of the immunohistochemical assays on these various tissues have been carried out. Analysis of these results, however, will take a number of months and can not be presented at this time. Comparison of the EGFR expression and intracellular localization profiles with those obtained from normal MEC undergoing branching alveolar morphogenesis *in vivo* and *in vitro* should help to define changes in EGFR levels and/or intracellular localization that take place during the development and progression of carcinogen-induced mammary tumors.

E. AIM 5. TEMPORAL ANALYSIS OF THE SIGNAL TRANSDUCTION PROTEINS THAT ARE TYROSINE PHOSPHORYLATED WHEN NORMAL MEC, VARIOUS TYPES OF MAMMARY STROMAL CELLS, AND CARCINOGEN-TRANSFORMED MEC ARE CULTURED IN THE PRESENCE OF EITHER EGF OR TGFα.

1. OVERVIEW

These studies were designed to compare the functional consequence of EGF- and TGFα-induced activation of the EGFR within normal MEC undergoing alveolar branching morphogenesis *in vitro* compared to that within normal rat mammary stromal cells as well as within rat mammary tumor cells transformed with the carcinogens n-methyl-nitrosourea or dimethylbenzanthracene. The main objective of these studies was to determine whether EGF and TGFα activated distinct signal transduction pathways during different phases of alveolar morphogenesis *in vitro*, and then to determine whether EGFR signaling in normal MEC differs from that of the different primary mammary stromal cells and/or carcinogen-exposed mammary tumor cells. Extensive effort was extended on adapting established methodologies to detect rapid signal transduction responses within the cultured normal MEC. Thus far, current approaches lack the sensitivity to examine rapid signal transduction events in normal MEC. It should be emphasized that last year, we were not able to detected rat EGFR or erbB2 in formalin-fixed and paraffin-embedded tissues or to detect all four rat erbB receptors by Western blot. Technical advancements are continually made to these protocols and one day soon we make the right adjustments to be able to examine relevant signaling responses in normal compared to malignant MEC. Success was, however, obtained in analyzing rapid signal transduction responses in serum-deprived normal rat mammary fibroblasts or rat breast adenocarcinoma (RBA) cells.

2. EXPERIMENTAL METHODS

EGFR Activation in Serum-Deprived Mammary Fibroblasts
(Ann Wohlhueter assisted in carrying out these experiments.)

Normal rat mammary fibroblasts (MFC) were cultured as a monolayer in 100 mm tissue culture plastic dishes in the presence of phenol red-free F12/DMEM medium with 10 % (v/v) fetal bovine serum (FBS) and 50 μg/ml gentamicin for 2 days, and then switched to F12/DMEM medium with 0.5 % (v/v) FBS and 50 μg/ml gentamicin for two consecutive 2 day

periods. After the serum-deprivation period, the MFC were washed twice with plain F12/DMEM medium and then exposed at 37°C to F12/DMEM medium with 1 mg/ml fatty acid-free bovine serum albumin supplemented with neither hrEGF or hrTGFα (NoGF), with 10 ng/ml EGF (EGF), or with 10 ng/ml TGFa (TGFα) for 5, 15, 60 or 120 minutes. MFC exposed to NoGF, EGF, or TGFα for 15 or 120 minutes were simultaneously exposed to 0 or 0.5 μM PD158780. Treatment medium was then removed, monolayers rinsed twice with ice-cold PBS, and then lysates prepared at 4°C in a 1 % (v/v) triton X-100 tyrosine phosphorylation lysis buffer that contained a cocktail of phosphatase and protease inhibitors. Lystates were separated on 4-12% gradient SDS-polyacrylamide gels, proteins transferred to Immobilon P membranes and then membranes sequentially immunoblotted with an anti-phosphotyrosine antibody (RC20-HRP), anti-phosphoMAPK, and anti-p44/42 MAPK antibodies as described below.

EGFR Activation in Serum-Deprived Rat Mammary RBA Cells
(Elizabeth Horn assisted in carrying out these experiments.)

Rat mammary RBA cells were obtained from ATCC (141) and cultured as a monolayer in 100 mm tissue culture plastic dishes in the presence of phenol red-free F12/DMEM medium with 10 % (v/v) fetal bovine serum (FBS) and 50 μg/ml gentamicin for 2 days, and then switched to F12/DMEM medium with 0.5 % (v/v) FBS and 50 μg/ml gentamicin for two consecutive 2-day periods. After the serum-deprivation period, the RBA cells were washed twice with plain F12/DMEM medium and then exposed at 37°C to either (1) epithelial NoGF medium with 0 or 0.5 μM PD158780 in DMSO at 0.1% (v/v) for 15 minutes or (2) epithelial EGF medium with 0 or 0.5 μM PD158780 in DMSO at 0.1% (v/v) for 15 minutes. Treatment medium was then removed, monolayers rinsed twice with ice-cold PBS, and then lysates prepared at 4°C in 1 % (v/v) triton X-100 tyrosine phosphorylation lysis buffer that contained a variety of phosphatases and proteases. Supernatant lysates were separated on 7.5% SDS-polyacrylamide gels, proteins were transferred to Immobilon P membranes, membranes were immunoblotted with a horseradish peroxidase conjugated recombinant anti-phosphotyrosine antibody (RC20-HRP), and tyrosine phosphorylated proteins were visualized using Amersham's ECL reagent. Membranes were then stripped with 2% (w/v) SDS and 100 mM 2-mercaptoethanol in 62.5 mM Tris, pH 6.7 at 50°C for 30 minutes, washed several times with PBS containing 0.1% (v/v) Tween 20, and immunoblotted with the polyclonal sheep anti-fusion protein antibody corresponding to a portion of the cytoplasmic tail of the human EGFR (UBI 06-129). EGFR immunoreactive proteins were detected using a horseradish peroxidase conjugated anti-sheep secondary antibody along with Amersham's ECL reagent. Exposed X-ray films were digitally scanned and processed as described above.

3. RESULTS

Rat mammary fibroblast cells (MFC) were successfully utilized to examine rapid signal transduction events including tyrosine phosphorylation protein profiles and MAPK phosphorylation in response to EGFR activation by EGF or TGFα in normal primary cells at passage 5. Specifically, the signal transduction cascades induced by 10 ng/ml of EGF or TGFα were compared using four different exposure times (5, 15, 60 and 120 minutes). The selective and potent inhibitor of the tyrosine kinase domain of the EGFR, PD158780, was then evaluated for its ability to block EGF- and TGFα-induced signal transduction. It should

be noted that MFC were simultaneously exposed to the appropriate EGFR ligand and the EGFR inhibitor. Both EGF and TGFα were effective at inducing the rapid (observable within 5 minutes, shorter times have yet to be evaluated) tyrosine phosphorylation of a ~170 kDa protein which co-migrates with an EGFR immunoreactive protein. The apparent phosphorylation of the EGFR was both concentration- [data not shown] and time-dependent [Figures 20-22]. EGF receptor phosphorylation was maximally induced with 10 ng/ml of either EGF or TGFα (higher concentration were not examined). Using either EGF receptor ligand at 10 ng/ml, induction of EGF receptor tyrosine phosphorylation peaked at 5 minutes [Figure 20], and appeared to be downregulated in a time- dependent manner from 15 minutes through the 120 minute treatment [Figures 20-22]. EGF and TGFα also induced a rapid (observable within 5 minutes, but shorter time points have yet to be tested) and sustained (through the 120 minute treatment) phosphorylation of mitogen activated protein kinase (MAPK) [Figures 20-22]. PD158780 was able block the apparent phosphorylation of the EGF receptor as well as the phosphorylation of MAPK [Figures 18-20]. Finally, these EGFR ligands also induced the phosphorylation of a variety of other proteins with distinct molecular weights. Additional studies are required to identify these tyrosine phosphorylated proteins and study the time-kinetics of their induction and down-regulation.

A study was then carried out to confirm that PD158780 was able to selectively inhibit EGFR-dependent tyrosine phosphorylation in rat mammary cells. Currently, our normal mammary primary culture system is not well suited to study rapid signal transduction responses. The main obstacles relate to the complex composition of the biologically active RBM and the abundance of RBM-derived proteins relative to MEC-derived proteins within the individual culture samples. In contrast, rapid signal transduction responses are easily studied in RBA cells cultured directly on tissue culture plastic in monolayer and synchronized by serum-starvation. This cell line was developed in the 1970's from a mammary adenocarcinoma induced in female Sprague-Dawley rats with the carcinogen DMBA (141).

A large variety of proteins were tyrosine phosphorylated when the serum-deprived RBA cells were exposed to NoGF medium for 15 minutes (Figure 23). A distinct subset of proteins were tyrosine phosphorylated when the RBA cells were exposed to EGF medium (Figure 23). PD158780 at 0.5 µM was able to inhibit EGF-induced tyrosine phosphosphorylation of the distinct subset of substrates (Figure 23) including the ~166 kDa protein that co-migrated with immunoreactive EGFR (Figure 23). In contrast, PD158780 was not able to inhibit ALV Medium-induced tyrosine phosphorylation in the RBA cells (Figure 23). Furthermore, certain proteins were hyperphosphorylated in the presence of NoGF medium EGF and 0.5 µM PD158780. This hyperphosphorylation may reflect the transmodulation of ErbB receptor activity and subsequent modulation of a tyrosine phosphatase in the absence of ligand-dependent activation. PD158780 would therefore be expected to block EGFR-dependent activation of the phosphatase and the subsequent apparent hyperphosphorylation. Furthermore, it should be noted that the RBA cells were serum-deprived, not serum-starved. EGF-like ligands may be present at low level in the serum. Alternatively, PD158780 may delay ErbB receptor internalization and down regulation which may result in an apparent increase in phosphorylated EGFR.

CONCLUSIONS

EGF and TGFα have been shown to regulate the development of normal mammary epithelial cells (MEC) and breast cancer cells. In addition, normal MEC, breast cancer cell lines as well as primary human breast cancers have been shown to express EGF and TGFα. These polypeptides have a three loop secondary structure generated when 6 evenly spaced cysteine residues form disulfide bonds. This structure allows these poplypeptides to selectively bind to the extracellular domain of EGFR, induce confirmational changes that result in receptor dimerization, activation of the cytoplasmic tyrosine kinase domain and autophosphorylation. Activated EGFR have been shown to phosphorylate kinases, phosphatases, adaptor proteins as well as other erbB receptors. EGFR, erbB2, erbB3 and erbB4 respresent the type I receptor tyrosine kinase receptor family also know as the erbB or EGFR family. Activated EGFR can homodimerize with EGFR or heterodimerize and transactivate erbB2, erbB3 or erbB4. High proportions of primary human breast cancers not only overexpress EGFR, one of the EGF-like ligands, and/or another erbB receptor, but these tumors usually respond poorly to endocrine therapy due to a lack of functional estrogen and/or progesterone receptors, and the patients generally have a short disease-free period and reduced overall chance for long term survival (1,51,61-67).

Unfortunately, the roles that the EGFR family and their ligands play in regulating the natural growth, differentiation, migration, invasion and survival of normal MEC from which breast cancer is derived are incompletely understood. It is therefore difficult to appreciate whether or not erbB receptors and their ligands are playing natural or pathologic roles in regulating breast cancer progression and metastasis. It seems that the answer to this question would have a profound impact on how to effectively regain control of erbB receptor positive breast cancer cells. With this point in mind, extensive effort during this three year award was focussed on more clearly defining the roles that erbB receptors and their ligands play in regulating the natural development of normal MEC.

Primary culture studies examining the effects of EGF and TGFα in normal MEC. PD158780, a selective inhibitor of the tyrosine kinase domain of EGFR, helped demonstrate that EGFR signaling was required for the proliferation and functional differentiation of immature MEC from days 0-7 of culture, and then for survival of terminally differentiated MEC.

Immunohistochemical and Western blot studies examined the expression and intracellular localization of EGFR, erbB2, erbB3 as well as erbB4 during the proliferation, differentiation and apoptosis of normal MEC. EGFR levels were expressed at high levels in non-functional MEC and MEC undergoing apoptosis whereas functionally differentiated MEC expressed relatively low levels of EGFR. In EGF medium, cultured MEC expressed peak levels of erbB2 and erbB3 from day 7-14 of culture, whereas erbB4 levels were only detected within MEC cultured for 7 and 10.5 days. MEC isolated from mammary glands expressed high levels of erbB2 and erbB3 in virgin rats and rats during pregnancy and involution, and relatively low levels during lactation, whereas erbB4 appeared to be uniformly expressed throughout the developmental stages analyzed.

In summary, the erbB receptor family appears to play dominant roles in regulating the growth, invasion and differentiation of immature and cytologically differentiated MEC during

branching alveolar morphogenesis, and the survival of terminally differentiated MEC. These findings help explain, in part, why human breast cancers that overexpress EGFR and/or other erbB receptors are so aggressive and often fatal.

In addition, immunohistochemical studies were also carried out on pre-malignant as well as malignant lesions within mammary glands of rats exposed to the carcinogen N-methyl-nitrosourea or dimethylbenzanthracene. Thus far, approximately 50% of the immunohistochemical assays are completely. Analysis of this data, however, will take a number of months and can not be presented at this time. Technical challenges during the first two years of this award prevented the initiation of these studies until the third year. It is hoped that by comparing the EGFR and erbB2 expression and intracellular localization profiles in pre-malignant and malignant lesions within those obtained from normal MEC undergoing branching alveolar morphogenesis *in vivo* and *in vitro* that we may better understand the roles (natural and/or pathologic) that erbB receptor levels play in the progression and metastasis of breast cancer.

It should also be noted that efforts to establish a mammary tumor progression model were more technically challenging then initially anticipated. This accomplishment although fundamental in nature represents a dramatic advance in the field of breast cancer research. This unique rat mammary tumor progression model system can directly interface with our existing primary culture model. Relevant pre-malignant and malignant MEC, isolated from carcinogen-exposed Sprague-Dawley rats, can now be cultured within the RBM in the presence of defined serum-free culture medium. During the next few months, carcinogen-exposed MEC will be used in the primary culture studies originally outlined in Aim 2 of this proposal to examine the biological activity of exogenous EGF and TGFα in pre-malignant MEC. Follow up studies will then determine whether the type and/or the magnitude of the effects induced in pre-malignant and/or malignant MEC are different than the effects observed in the normal MEC. Finally, it is hoped that identification of early and/or progressive changes during the progression of breast cancer will identify new targets that can be exploited for the development of effective breast cancer prevention and treatment strategies.

FIGURE LEGENDS

Figure 1. Effect of PD158780 on Viable Cell Number and Colony Survival. Panel A. Epithelial cell growth when MEC were cultured for 21 days within the RBM in medium containing no growth factors (NoGF Medium), 10 ng/ml EGF (EGF Medium) or 10 ng/ml TGF-α (TGF-α Medium). MEC cultured in NoGF Medium were exposed to 0, 0.05, 0.5 or 5 μM PD158780 from day 0-21. Viable cell number within individual culture wells was monitored using the MTT assay at different times during the 21 day culture period. Each point represents the mean ± the SEM obtained from triplicate culture wells. **Panel B.** Colony number in the above experiment was evaluated at day 21 of the study. A significant decrease (p<0.05) in colony number was found when MEC were cultured in NoGF Medium with 5 μM PD158780 (*). All other groups did not differ from the NoGF control. Data points represent the mean colony count from triplicate culture wells.

Figure 2. Effect of Continuous Versus Short Term Exposure to PD158780 on Viable Cell Number. Epithelial cell growth when MEC were cultured for 21 days within the RBM in medium containing no growth factors (NoGF), 10 ng/ml EGF (EGF) or 10 ng/ml TGF-α (TGF). PD158780 was present either from day 0-21 or added at day 17.5-21. Viable cell number within individual culture wells was monitored using the MTT assay. Each bar represents the mean ± the SEM obtained from triplicate culture wells. There was a statistically significant difference (*) between the solvent control and PD158780 treatment groups with continuous presence, but not when PD158780 was added at day 17.5 (p<0.05).

Figure 3. Effect of Short Term Exposure to PD158780 on Viable Cell Number and Casein Accumulation in MEC Cultured for up to 10.5 Days. Panel A. Epithelial cell growth when MEC were cultured for up to 10.5 days within the RBM in medium containing no growth factors (NoGF Medium), 10 ng/ml EGF (EGF Medium) or 10 ng/ml TGF-α (TGF-α Medium). MEC were exposed to 0 or 0.5 μM PD158780 from day 0-3.5, day 3.5-7, or from day 7-10.5. Viable cell number within individual culture wells was monitored using the MTT assay at different times during the 10.5 day culture period. There was a statistically significant difference (*) between the control and PD158780 treatment groups at day 3.5 (in EGF and TGFα groups) and day 7 (all groups), but not at day 10.5. **Panel B.** Casein accumulation in the experiment described in Figure 3A was monitored within individual culture wells at different times during the 10.5 day culture period by ELISA using a polyclonal antibody raised against the entire family of casein milk proteins. There was a statistically significant difference (*) between the control and PD158780 treatment groups at day 7 in NoGF medium, at day 3.5 and day 7 in EGF medium, and at all three time points in TGFα medium (p<0.05). Each bar represents the mean ± the SEM obtained from triplicate culture wells.

Figure 4. Effect of Short Term Exposure of PD158780 on Viable Cell Number and Casein Accumulation in MEC Cultured for up to 21 Days. Panel A. Epithelial cell growth was evaluated in MEC cultured for up to 21 days within the RBM in NoGF, EGF or TGFα medium. MEC were exposed to 0 or 0.5 μM PD158780 from day 3.5-7, day 10.5-14, or from day 17.5-21. Viable cell number within individual culture wells was monitored using the MTT assay at different times during the 21 day culture period. There was a statistically significant

difference between the control and PD158780 treatment groups at day 7 (EGF and TGFα medium only), and at day 21 in TGFα medium. **Panel B.** Casein accumulation in the experiment described in Figure 4A was monitored by ELISA using the polyclonal antibody raised against the entire family of casein milk proteins. There was a statistically significant difference (*) between the control and PD158780 treatment groups at day 7 in EGF and TGFα medium, and at day 21 in TGFα medium ($p < 0.05$). Each bars represents the mean ± the SEM obtained from triplicate culture wells.

Figure 5. Apoptosis in Cultured MEC. Panels A-D. Light photomicrographs of representative intact healthy **(A, C)** and apoptotic **(B, D)** epithelial organoids cultured for 21 days within the RBM in EGF medium. **Panels E-F.** Electron photomicrographs of representative healthy **(E)** and late stage apoptotic **(F)** epithelial organoids. **Panels G-H.** Light photomicrographs of representative sections of healthy **(G)** and apoptotic **(H)** epithelial organoids analyzed utilizing the *in situ* apoptosis detection assay (TUNEL Assay). Note the absence **(G)** and presence of apoptotic DNA **(H)** visualized using a brown chromogen. Epithelial organoids undergoing apoptosis accumulated moderate to abundant quantities of hematoxylin-stained condensed DNA within their lumen **(H-L)**. **Panels I-J.** Immunohistochemical detection of EGFR within representative epithelial organoids undergoing apoptosis using the affinity-purified rabbit anti-peptide EGFR antibody (SC-03) alone **(I)** or the SC-03 antibody pre-mixed with the EGFR immunizing peptide **(J)**. EGFR staining was visualized with the brown chromogen DAB. **Panels K-L.** Immunohistochemical detection of erbB2 within representative epithelial organoids undergoing apoptosis using the affinity-purified rabbit anti-peptide erbB2 antibody (SC-284) alone **(I)** or the SC-284 antibody pre-mixed with the erbB2 immunizing peptide **(J)**. ErbB2 staining was visualized with the brown chromogen DAB. Magnification bars in panels E-F represent 1 μm whereas those in G-H represent 10 μm.

Figure 6. Effect of Short Term Exposure of PD158780 on the Expression/Accumulation of Casein Isoforms. Casein isoform distribution was analyzed in MEC cultured for 7, 14 or 21 days in NoGF, EGF or TGF medium by Western blot with a polyclonal antibody raised against the entire family of rat casein isoforms. MEC cultured for 7, 14 or 21 days in NoGF (lanes 1 and 2), EGF (lanes 3 and 4), or TGFα (lanes 5 and 6) medium were exposed to 0 (-) or 0.5 μM (+) PD158780 from days 3.5-7, days 10.5-14 or days 17.5-21, respectively. Day 7 lysates were loaded to represent 7500 cells/lane, whereas day 14 and day 21 lysates were loaded to represent 10,000 cells/lane. Molecular weight markers in kDa are provided along the left side of the images.

Figure 7. Effect of Short Term Exposure of PD158780 on the Expression/Accumulation of Casein Isoforms. Casein isoform distribution was analyzed in MEC cultured for up to 21 days in NoGF medium using a casein Western blot protocol. **Panel A** represents lysates of MEC cultured for 3.5, 7 or 10.5 days and exposed to 0 (-) or 0.5 μM (+) PD158780 from days 0-3.5, days 3.5-7 or days 7-10.5, respectively. **Panel B** represents lystates of MEC cultured for 14, 17.5 or 21 days and exposed to 0 (-) or 0.5 μM (+) PD158780 from days 10.5-14, days 14-17.5 or days 17.5-21, respectively. Day 3.5-10.5 lysates were loaded to represent 4000 cells/lane while day 14-21 lysates were loaded to represent 10,000 cells/lane. Molecular weight markers in kDa are provided along the left side of the images.

Figure 8. Effect of Short Term Exposure of PD158780 on the Expression/Accumulation of Casein Isoforms. Casein isoform distribution was analyzed in MEC cultured for up to 21 days in EGF medium using a casein Western blot protocol. **Panel A** represents lysates of MEC cultured for 3.5, 7 or 10.5 days and exposed to 0 (-) or 0.5 μM (+) PD158780 from days 0-3.5, days 3.5-7 or days 7-10.5, respectively. **Panel B** represents lystates of MEC cultured for 14, 17.5 or 21 days and exposed to 0 (-) or 0.5 μM (+) PD158780 from days 10.5-14, days 14-17.5 or days 17.5-21, respectively. Day 3.5 lysates were loaded to represent 5,000 cells/lane while day 7-21 lysates were loaded to represent 10,000 cells/lane. Molecular weight markers in kDa are provided along the left side of the images.

Figure 9. Effect of Short Term Exposure of PD158780 on the Expression/Accumulation of Casein Isoforms. Casein isoform distribution was analyzed in MEC cultured for up to 21 days in TGFα medium using a casein Western blot protocol. **Panel A** represents lysates of MEC cultured for 3.5, 7 or 10.5 days and exposed to 0 (-) or 0.5 μM (+) PD158780 from days 0-3.5, days 3.5-7 or days 7-10.5, respectively. **Panel B** represents lysates of MEC cultured for 14, 17.5 or 21 days and exposed to 0 (-) or 0.5 μM (+) PD158780 from days 10.5-14, days 14-17.5 or days 17.5-21, respectively. Day 3.5 lysates were loaded to represent 5,000 cells/lane while day 7-21 lysates were loaded to represent 10,000 cells/lane. Molecular weight markers in kDa are provided along the left side of the images.

Figure 10. **Epithelial expression of EGFR during different stages of *in vitro* development of MEC cultured in EGF Medium.** Epithelial lysates of MEC cultured for 0 (2 hours), 3.5, 7, 10.5, 14, 17.5 or 21 days in EGF Medium were analyzed using a Western blot protocol with a polyclonal sheep anti-EGFR antibody. **Panel A.** Day 3.5, 7, 10.5, 14, 17.5 and 21 lysate samples were evaluated using 10 μg of protein per lane. **Panel B.** Equal volumes (25 μl per lane) of each of the following lysates were also evaluated: Day 0 (8.7 μg/lane), day 3.5 (16.7 μg/lane), day 7 (63.3 μg/lane), day 10.5 (65.7 μg/lane), day 14 (73.1 μg/lane), day 17.5 (85 μg/lane, day 21 #1 (101 μg/lane) and day 21 #2 (76.9 μg/lane).

Figure 11. **Epithelial expression of EGFR during different stages of *in vitro* development of MEC cultured in TGFα Medium.** Epithelial lysates of MEC cultured for 0 (2 hours), 3.5, 7, 10.5, 14, 17.5 or 21 days in TGFα Medium were analyzed using a Western blot protocol with a polyclonal sheep anti-EGFR antibody. **Panel A.** Day 3.5, 7, 10.5, 14, 17.5 and 21 lysate samples were evaluated using 10 μg of protein per lane. **Panel B.** Equal volumes (25 ml per lane) of each of the following lysates were also evaluated: Day 0 (8 μg/lane), day 3.5 (26.5 μg/lane), day 7 (83.4 μg/lane), day 10.5 (76.4 μg/lane), day 14 (69 μg/lane), day 17.5 (86 μg/lane), day 21 #1 (82.7 μg/lane) and day 21 #2 (111.6 μg/lane).

Figure 12. **Epithelial expression of EGFR during different stages of *in vitro* development of MEC cultured in NoGF Medium.** Epithelial lysates of MEC cultured for 0 (2 hours), 3.5, 7, 10.5, 14, 17.5 or 21 days in NoGF Medium were analyzed using a Western blot protocol with a polyclonal sheep α-EGFR antibody. **Panel A.** Equal volumes (25 μl per lane) of each of the following lysates were also evaluated: Day 0 (8.8 μg/lane), day 3.5 (14.7 μg/lane), day 7 (18.8 μg/lane), day 10.5 (19.6 μg/lane), day 14 (26.4 μg/lane), day 17.5 (46 μg/lane, day 21 #1 (91.3 μg/lane) and rat liver lysate (RLL) (41 μg/lane).

Figure 13. EGFR receptor expression with MEC cultured in EGF medium. EGFR intracellular localization within representative sections of mammary epithelial organoids cultured in EGF medium for 0 day actually 2 hours (A), 3.5 days (B), 7 days (C), 14 days (D), or 21 days (E). The immunohisotchemistry assays were carried out using a polyclonal rabbit anit-peptide EGFR antibody (SC-03) and EGFR visualized with the brown chromogen DAB.

Figure 14. **Epithelial expression of EGFR during different stages of mammary gland development *in vivo*.** Trizol samples were prepared from MEC isolated from three sets of 50-55 day-old virgin rats (V), rats at day 14-16 of pregnancy (P), rats at day 6-7 of lactation (L6 or L7), rats at day 21 of lactation (L21), and rats at day 7 of involution (I7). Lysates were also prepared from MEC isolated from rats at day 10 of lactation (L10) as well as day 15 of lactation (L15). Finally, samples were prepared from MEC isolated from rats at day 3 of involution (I3). Samples from rats at day 21 of lactation actually also represent 12 hour of involution since 21 day-old pups were removed from their mothers 12 hours prior to excising the mammary glands from the rats. Samples were evaluated at 50 μg of protein per lane using a Western blot protocol with a polyclonal sheep α-EGFR antibody. Panels A, B and C represent samples obtained from three different sets of developmental samples.

Figure 15. EGFR receptor expression during mammary gland development *in vivo*. EGFR intracellular localization within representative sections of mammary glands from 50-54 day-old virgin rats **(Panels A1-A8)**, rats at day 7 or day 14-16 of pregnancy **(Panels B1-B4)**,

rats at day 5 or day 15 of lactation (**Panel C1-C4**), rats at day 21 of lactation (**Panels C5-C8**), rats at day 3-4 of involution (**Panels D1-D4**), or rats at day 6-10 of involution (**Panels D5-D8**). The immunohisotchemistry assays were carried out using a polyclonal rabbit anit-peptide EGFR antibody (SC-03) and EGFR visualized with the brown chromogen DAB. **A1:** Alveolar cluster from day 50 virgin rat. **A2:** Terminal end bud from day 54 virgin rat. **A3:** Duct surrounded by fibroblasts from day 54 virgin rat. **A4:** Adipocytes from day 54 virgin rat. **A5:** Fibroblasts surrounding alveolar cluster from day 54 virgin rat. **A6:** Alveolar cluster from day 54 virgin rat. **A7:** Duct surrounded by fibroblasts from day 54 virgin rat. **A8:** Adipocytes from day 54 virgin rat. **B1:** Alveolar cluster from day 7 pregnant rat. **B2:** Alveolar cluster from day 14-16 pregnant rat. **B3:** Duct surrounded by fibroblasts from day 7 pregnant rat. **B4:** Adipocytes from day 14-16 pregnant rat. **C1:** Alveolus from day 7 lactating rat. **C2:** Alveolar cluster from day 15 lactating rat. **C3:** Duct surrounded by fibroblasts from day 5 lactating rat. **C4:** Adipocytes from day 7 lactating rat. **C5:** Alveolar cluster from day 21 lactating/12 hour involuting rat. **C6:** Alveolar cluster from day 21 lactating/12 hour involuting rat. **C7:** Duct surrounded by fibroblasts from day 21 lactating/12 hour involuting rat. **C8:** Adipocytes from day 21 lactating/12 hour involuting rat. **D1:** Alveolar cluster near the nipple (within 2 cm) from day 3 involuting rat. **D2:** Alveolar cluster near nipple (within 2 cm) from day 3 involuting rat. **D3:** Duct near nipple (within 2 cm) from day 3 involuting rat. **D4:** Adipocytes near nipple (within 2 cm) from day 3 involuting rat. **D5:** Alveolar cluster away from nipple (> 2 cm) from day 3-4 involuting rat. **D6:** Alveolar cluster away from nipple (> 2 cm) from day 4 involuting rat. **D7:** Fibroblasts away from nipple (>2cm) from day 4 involuting rat. **D8:** Adipocytes away from nipple (within 2 cm) from day 4 involuting rat. **D9:** Alveolar cluster from day 6 involuting rat. **D10:** Alveolar cluster from day 10 involuting rat. **D11:** Duct surrounded by fibroblasts from day 10 involuting rat. **D12:** Adipocytes from day 6 involuting rat. Clear arrows indicate distinct apical plasma membrane staining, large arrows indicate nuclear/supra-nuclear staining. Magnification bars equal 10 μm.

Figure 16. EGFR, erbB2, erbB3 and erbB4 Expression in MEC Cultured for up to 21 Days in EGF Medium. Epithelial lysates were prepared from MEC cultured for 0, 3.5, 7, 10.5, 14, 17.5 or 21 days in EGF Medium. **Panel A.** EGFR detection in day 3.5, 7, 10.5, 14, 17.5 and 21 lysates with 10 μg of protein loaded per lane. Western blot assay performed using a polyclonal sheep anti-EGFR antibody (UBI 06-129). **Panel B.** EGFR detection in each of the following samples loaded with 25 μl of lysate per lane (protein concentration loaded per lane): Day 0 (8.7 μg/lane), day 3.5 (16.7 μg/lane), day 7 (63.3 μg/lane), day 10.5 (65.7 μg/lane), day 14 (73.1 μg/lane), day 17.5 (85 μg/lane, day 21 #1 (101 μg/lane) and day 21 #2 (76.9 μg/lane). Western blot assay was performed using a polyclonal sheep anti-EGFR antibody (UBI 06-129). **Panel C.** ErbB2 detection in day 3.5, 7, 10.5, 14, 17.5 and 21 lysates with 10 μg protein loaded per lane. In addition, day 0 lysate was analyzed with 5 μg protein loaded per lane and rat liver lysate evaluated at 20 μg/lane. Western blot assay was performed using a polyclonal rabbit anti-peptide erbB2 antibody (SC-284). **Panel D.** ErbB3 detection in day 3.5, 7, 10.5, 14, 17.5 and 21 lysates with 10 μg protein loaded per lane. Day 0 lysate was analyzed with 5 μg of protein loaded per lane. Western blot assay was performed using a polyclonal rabbit anti-peptide erbB3 antibody (SC-285). **Panel E.** ErbB4 detection in day 3.5, 7, 10.5, 14, 17.5 and 21 lysates with 10 μg of protein loaded per lane. In addition, day 0 lysate was analyzed with 5 μg protein loaded per lane and rat liver lysate evaluated at 20 μg/lane. Western blot assay was performed using a polyclonal rabbit anti-peptide erbB4 antibody (SC-283).

Figure 17. Epithelial expression of EGFR, erbB2, erbB3 and erbB4 during different stages of mammary gland development *in vivo.* Trizol samples were prepared from MEC isolated from three sets of 50-55 day-old virgin rats (V), rats at day 14-16 of pregnancy (P), rats at day 6-7 of lactation (L6 or L7), rats at day 21 of lactation (L21), and rats at day 7 of involution (I7). Lysates were also prepared from MEC isolated from rats at day 10 of lactation (L10) as well as day 15 of lactation (L15). Trizol samples were also prepared from MEC isolated from one set of rats at day 3 of involution (I3). All samples were evaluated at 50 μg of protein loaded per lane. **Panel A.** EGFR detection using a purified polyclonal sheep α-EGFR antibody (UBI 06-129). Isotype control analyzed using sheep IgG (Sheep IgG). **Panel B.** ErbB2 detection using an affinity-purified polyclonal rabbit anti-peptide erbB2 antibody (SC-284). Peptide competition analyzed using the polyclonal rabbit anti-peptide erbB2 antibody pre-mixed with the immunizing erbB2 peptide (SC-284 + SC284P competition). **Panel C.** ErbB3 detection using an affinity-purified polyclonal rabbit anti-peptide erbB3 antibody (SC-285). Peptide competition evaluated using the polyclonal rabbit anti-peptide erbB3 antibody pre-mixed with the immunizing erbB3 peptide (SC-285 + SC285P competition). **Panel D.** ErbB4 detection using an affinity-purified polyclonal rabbit anti-peptide erbB4 antibody (SC-283). Peptide competition evaluated using the polyclonal rabbit anti-peptide erbB4 antibody pre-mixed with the immunizing erbB4 peptide (SC-283 + SC283P competition). Isotype control for erbB2, erbB3 and erbB4 using rabbit IgG as an isotype control for SC-284, SC-285 and SC-283 (Rabbit IgG).

Figure 18. ErbB2 receptor expression in MEC cultured in EGF medium. ErbB2 intracellular localization within representative sections of mammary epithelial organoids cultured in EGF medium for 2 hours (A), 3.5 days (B), 7 days (C), 14 days (D), or 21 days (E). The immunohistochemistry assays were carried out using an affinity-purified polyclonal rabbit anit-peptide erbB2 antibody (SC-284) and erbB2 visualized with the brown chromogen DAB.

Figure 19. ErbB2 receptor expression during mammary gland development *in vivo.* ErbB2 intracellular localization within representative sections of mammary glands from 50-54 day-old virgin rats (Panels A1-A8), rats at day 7 or day 14-16 of pregnancy (Panels B1-B4), rats at day 5 or day 15 of lactation (Panel C1-C4), rats at day 21 of lactation (Panels C5-C8), rats at day 3-4 of involution (Panels D1-D8), or rats at day 6-10 of involution (panels D9-D12). The immunohisotchemistry assays were carried out using an affinity-purified polyclonal rabbit anit-peptide erbB2 antibody (SC-284) and erbB2 visualized with the brown chromogen DAB. **A1:** Alveolar cluster from day 50 virgin rat. **A2:** Terminal end bud from day 54 virgin rat. **A3:** Duct surrounded by fibroblasts from day 54 virgin rat. **A4:** Adipocytes from day 54 virgin rat. **A5:** Fibroblasts surrounding alveolar cluster from day 54 virgin rat. **A6:** Alveolar cluster from day 54 virgin rat. **A7:** Duct surrounded by fibroblasts from day 54 virgin rat. **A8:** Adipocytes from day 54 virgin rat. **B1:** Alveolar cluster from day 7 pregnant rat. **B2:** Alveolar cluster from day 14-16 pregnant rat. **B3:** Duct surrounded by fibroblasts from day 7 pregnant rat. **B4:** Adipocytes from day 14-16 pregnant rat. **C1:** Alveoli from day 7 lactating rat. **C2:** Alveolar cluster from day 15 lactating rat.**C3:** Duct surrounded by fibroblasts from day 5 lactating rat. **C4:** Adipocytes from day 7 lactating rat. **C5:** Alveolar cluster from day 21 lactating/12 hour involuting rat. **C6:** Alveolar cluster from day 21 lactating/12 hour involuting rat. **C7:** Duct surrounded by fibroblasts from day 21 lactating/12 hour involuting rat. **C8:**

Adipocytes from day 21 lactating/12 hour involuting rat. **D1**: Alveolar cluster away from nipple (>2 cm) from day 3 involuting rat. **D2**: Alveolar cluster away from nipple (>2 cm) from day 3 involuting rat. **D3**: Duct away from nipple (>2 cm) from day 3 involuting rat. **D4**: Adipocytes away from nipple (>2 cm) from day 3 involuting rat. **D5**: Alveolar cluster away from nipple (>2 cm) from day 3-4 involuting rat. **D6**: Alveolar cluster near nipple (within 2 cm) from day 4 involuting rat. **D7**: Fibroblasts near nipple (within 2 cm) from day 4 involuting rat. **D8**: Adipocytes near nipple (within 2 cm) from day 4 involuting rat. **D9**: Alveolar cluster from day 6 involuting rat. **D10**: Alveolar cluster from day 10 involuting rat. **D11**: Duct surrounded by fibroblasts from day 10 involuting rat. **D12**: Adipocytes from day 6 involuting rat. Clear arrows indicate distinct apical plasma membrane staining, large arrows indicate nuclear/supra-nuclear staining. Magnification bars equal 10 μm.

Figure 20. EGFR-dependent tyrosine phosphorylation of serum-deprived normal rat mammary fibroblasts activated with EGF or TGFα. Serum starved mammary fibroblasts were activated for 5 minutes or 1 hour with F12/DMEM medium alone (NoGF), F12/DMEM medium with 10 ng/ml EGF (EGF) or F12/DMEM medium with 10 ng/ml TGFα. Whole cell lysates were prepared and 10 mg of each sample loaded per lane. Molecular weight markers are provided in kDa to the left of each image. **Top Panel.** Western blot image using a horseradish peroxidase conjugated recombinant anti-phosphotyrosine antibody, RC20-HRP. **Middle Panel.** Western blot image using a polyclonal antibody specific for the tyrosine phosphorylated MAPK on the same membrane used for panel A after stripping away the RC-20 antibody. **Bottom Panel.** Western blot image using a polyclonal antibody against total p44/p42 MAPK on the same membrane used above after stripping the phospho-MAPK antibody.

Figure 21. PD158780 inhibition of EGFR-dependent tyrosine phosphorylation of serum-deprived normal rat mammary fibroblasts activated with EGF or TGFα. Serum starved mammary fibroblasts were activated for 15 minutes with F12/DMEM medium alone (NoGF) without or with 0.5 μM PD158780, F12/DMEM medium with 10 ng/ml EGF (EGF) without or with 0.5 μM PD158780, or F12/DMEM medium with 10 ng/ml TGFα without or with 0.5 μM PD158780. Whole cell lysates were prepared and 10 mg of each sample loaded per lane. Sequential Western blot assays were performed on samples run and transferred to one membrane. Molecular weight markers are provided in kDa to the left of each image. **Top Panel.** Western blot image using a horseradish peroxidase conjugated recombinant anti-phosphotyrosine antibody, RC20-HRP. **Middle Panel.** Western blot image using a polyclonal antibody specific for the tyrosine phosphorylated MAPK on the same membrane used for panel A after stripping away the RC-20 antibody. **Bottom Panel.** Western blot image using a polyclonal antibody against total p44/p42 MAPK on the same membrane used above after stripping the phospho-MAPK antibody.

Figure 22. PD158780 inhibition of EGFR-dependent tyrosine phosphorylation of serum-deprived normal rat mammary fibroblasts activated with EGF or TGFα. Serum starved mammary fibroblasts were activated for 2 hours with F12/DMEM medium alone (NoGF) without or with 0.5 μM PD158780, F12/DMEM medium with 10 ng/ml EGF (EGF) without or with 0.5 μM PD158780, or F12/DMEM medium with 10 ng/ml TGFα without or with 0.5 μM PD158780. Whole cell lysates were prepared and 10 mg of each sample loaded per lane. Sequential Western blot assays were performed on samples run and transferred to one

membrane. Molecular weight markers are provided in kDa to the left of each image. **Top Panel.** Western blot image using a horseradish peroxidase conjugated recombinant anti-phosphotyrosine antibody, RC20-HRP. **Middle Panel.** Western blot image using a polyclonal antibody specific for the tyrosine phosphorylated MAPK on the same membrane used for panel A after stripping away the RC-20 antibody. **Bottom Panel.** Western blot image using a polyclonal antibody against total p44/p42 MAPK on the same membrane used above after stripping the phospho-MAPK antibody.

Figure 23. PD158780 selectively inhibits EGF-induced tyrosine phosphorylation in serum-deprived RBA cells. Serum-deprived rat breast adenocarcinoma cell line, RBA, was stimulated for 15 minutes with NoGF Medium, NoGF Medium + 0.5 μM PD158780, EGF Medium, or EGF medium + 0.5 μM PD158780. Lysates were prepared from each of these four groups and analyzed using 25 μg of protein loaded per lane. Western blot protocols were carried out using a horseradish conjugated recombinant anti-phosphotyrosine antibody (RC20-HRP). Molecular weight markers in kDa are provided along the left side of the image.

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Figure 1.

Darcy, Kathleen M.

A.

B.

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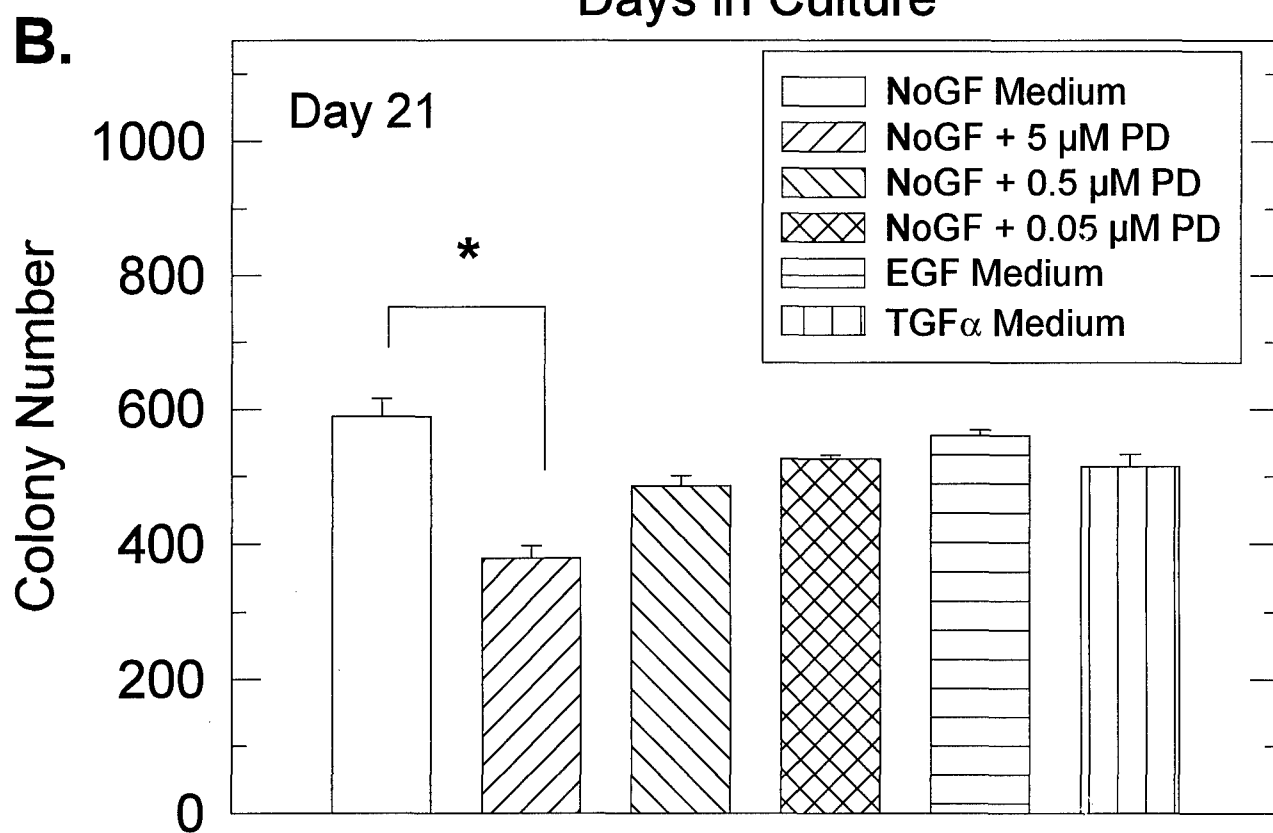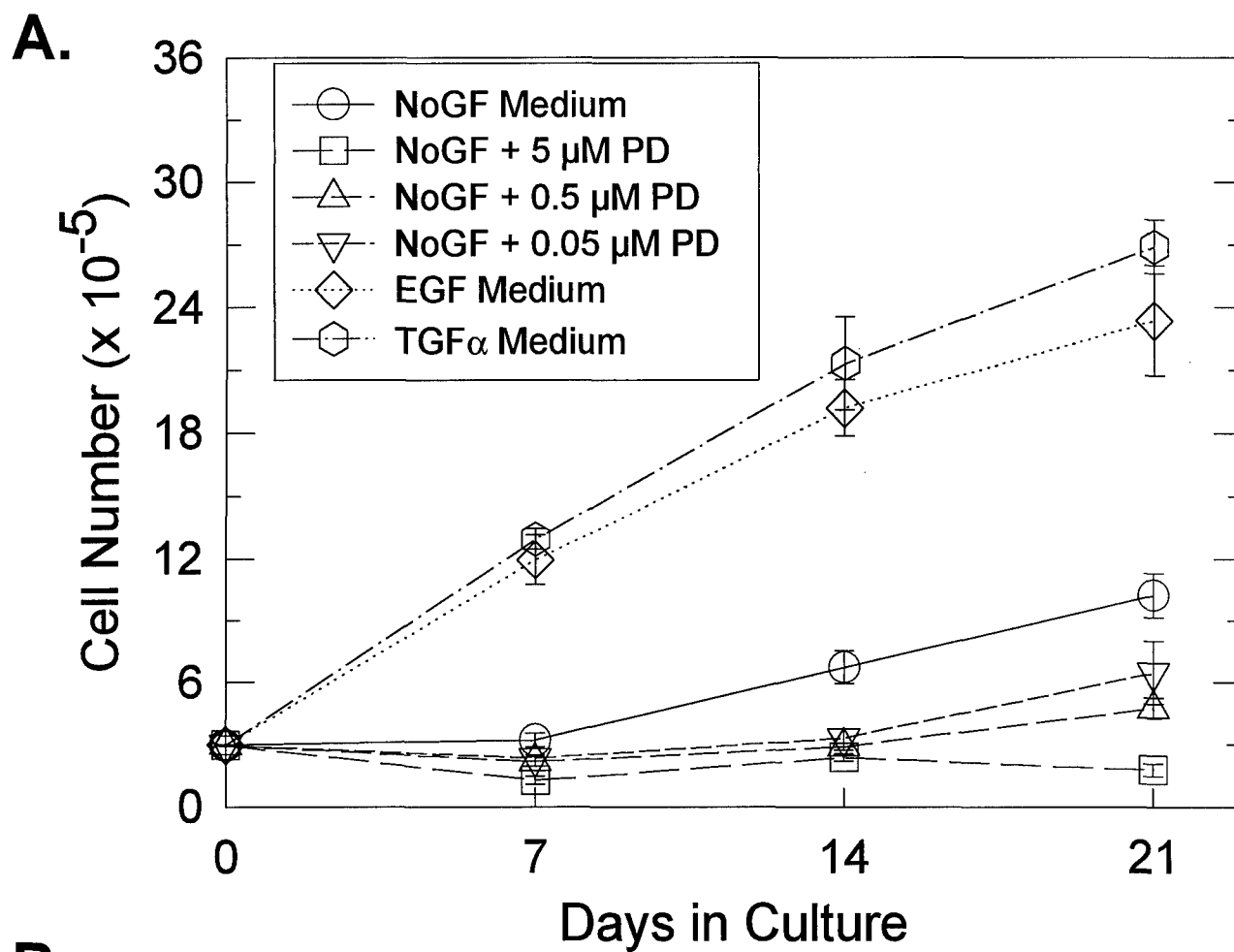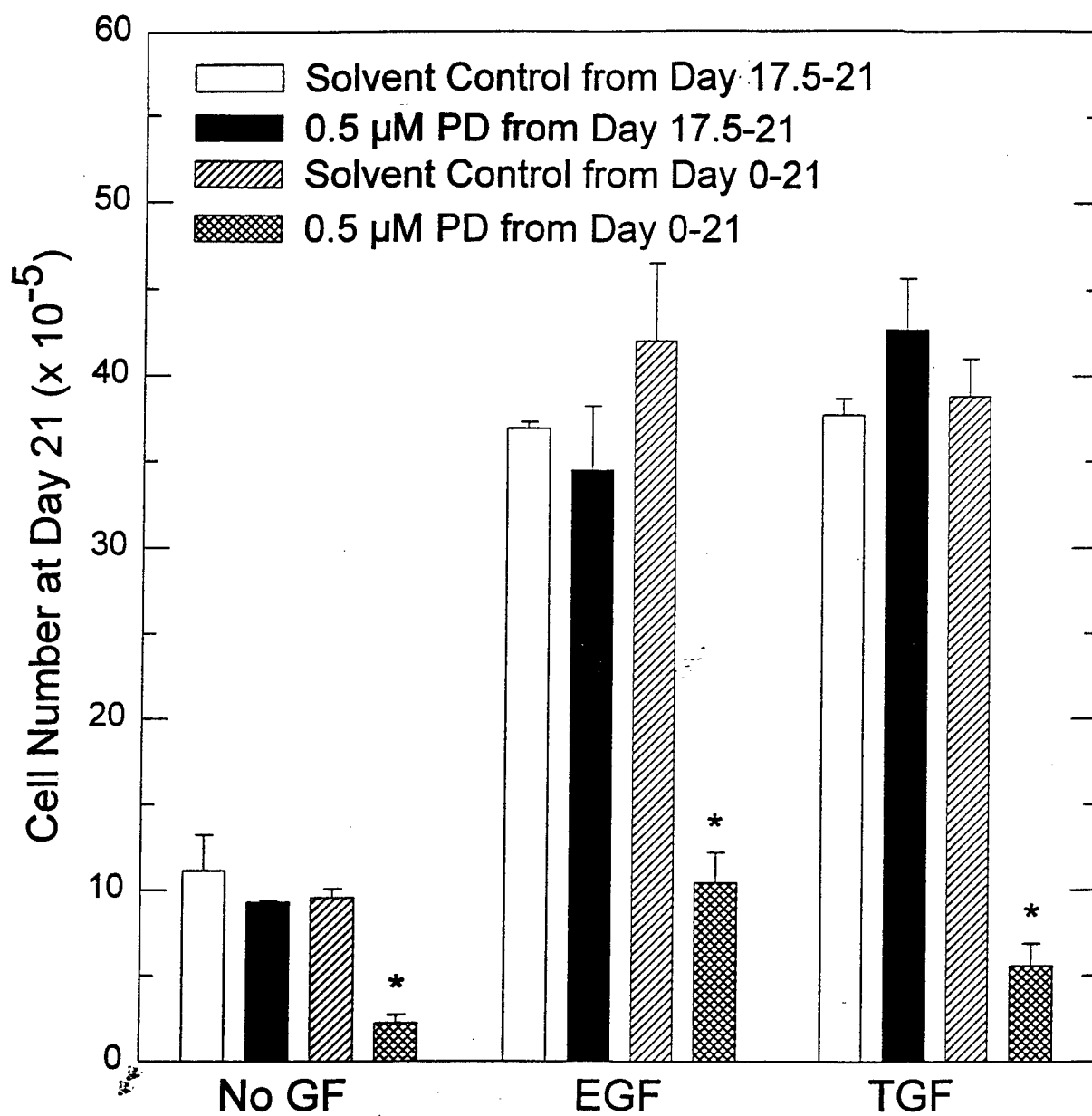
Figure 2.

Figure 3.

Darcy, Kathleen M.

A.

Legend:
☐ No PD158780
■ 0.5 µM PD158780 from day 0-3.5, 3.5-7 or 7-10.5

Y-axis: Cell Number (x 10^{-5})
X-axis: Days in Culture

NoGF Medium, EGF Medium, TGF Medium

B.

Legend:
☐ No PD158780
■ 0.5 µM PD158780 from day 0-3.5, 3.5-7 or 7-10.5

Y-axis: Casein (ng/10^5 cells)
X-axis: Days in Culture

NoGF Medium, EGF Medium, TGF Medium

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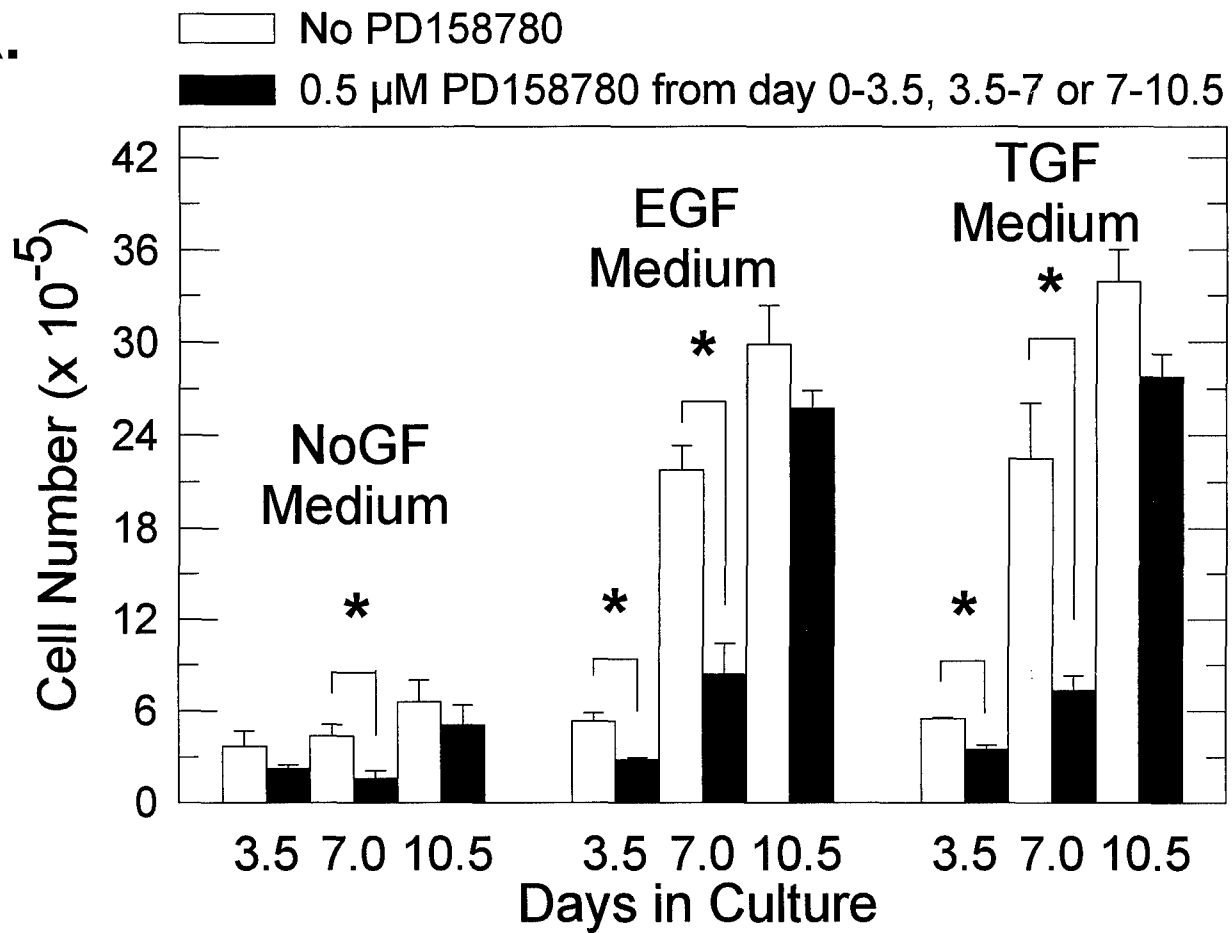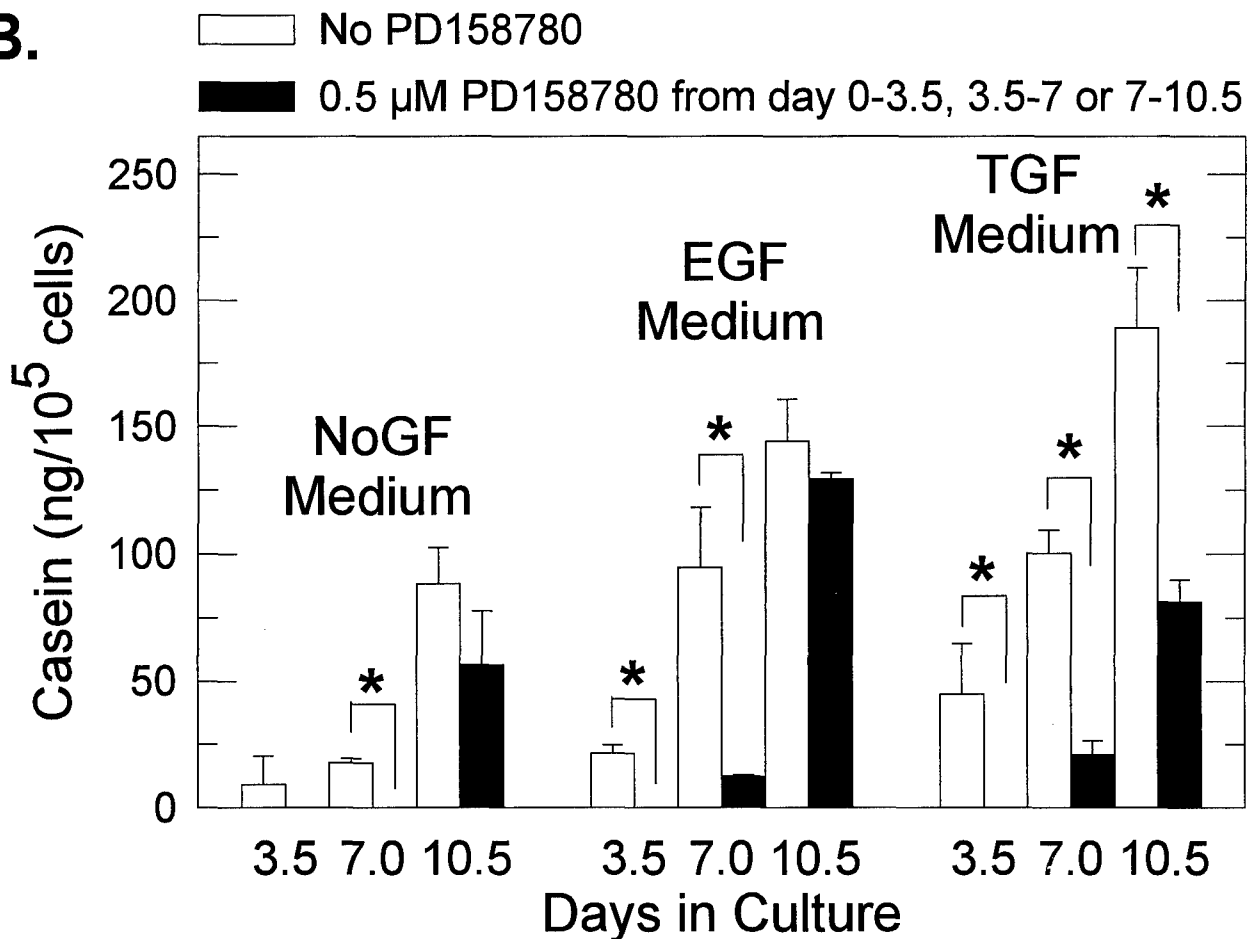
Figure 4.

Darcy, Kathleen M.

A.

□ NoPD158780

■ 0.5 µM PD15870 from day 3.5-7, 10.5-14 or 17.5-21

EGF Medium

TGF Medium

NoGF Medium

Cell Number (x 10^{-5})

Days in Culture

B.

□ NoPD158780

■ 0.5 µM PD158780 from day 3.5-7, 10.5-14 or 17.5-21

EGF Medium

TGF Medium

NoGF Medium

Casein (ng/10^5 cells)

Days in Culture

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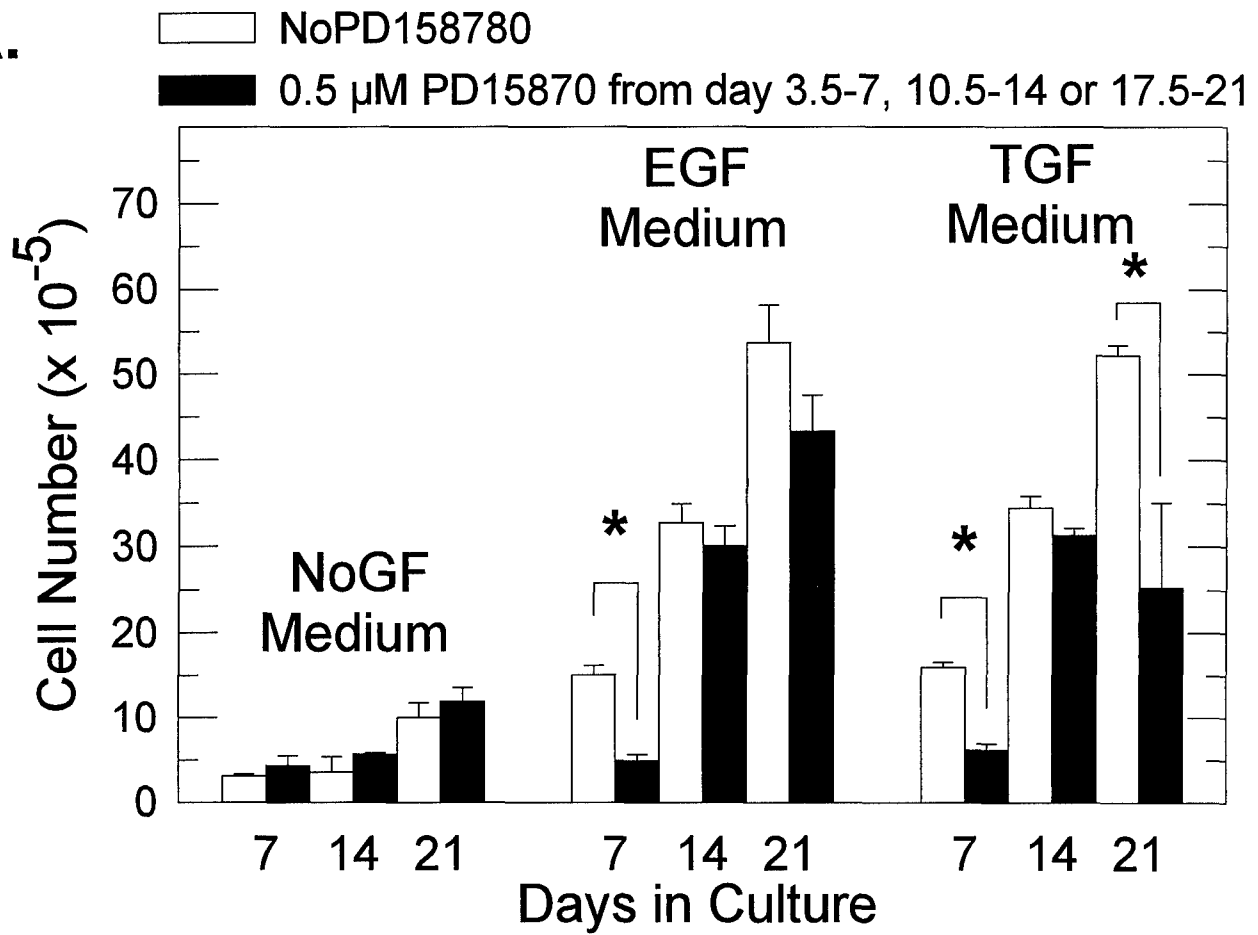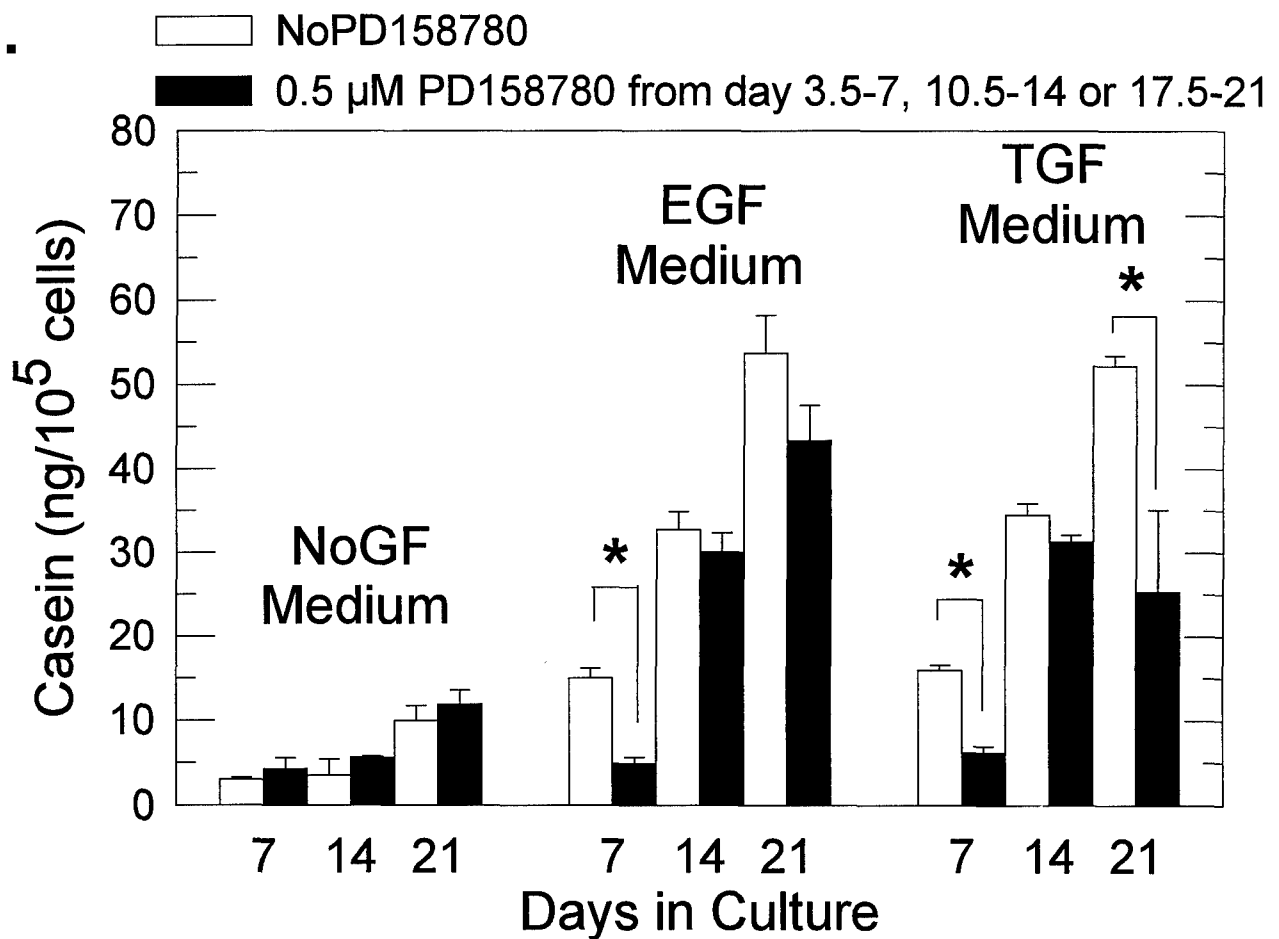
FIGURE 5.

Figure 6.

Figure 7.

A.

| 3.5 | 7 | 10.5 |

- + - + - +

45 —
31 —
21 —

α₁
α₂
K²
β
γ

B.

| 14 | 17.5 | 21 |

- + - + - +

45 —
31 —
21 —

α₁
α₂
K²
β
γ

Figure 8.

A.

B.

Figure 9.   Darcy, Kathleen M.
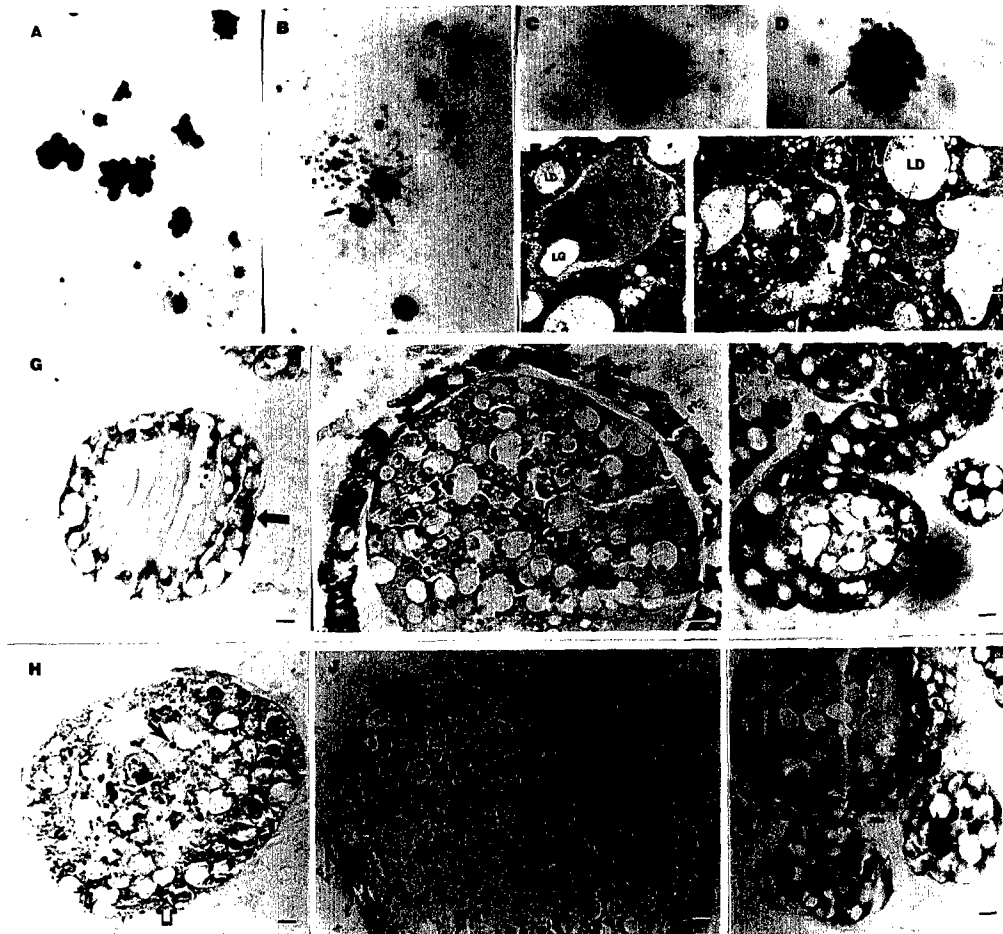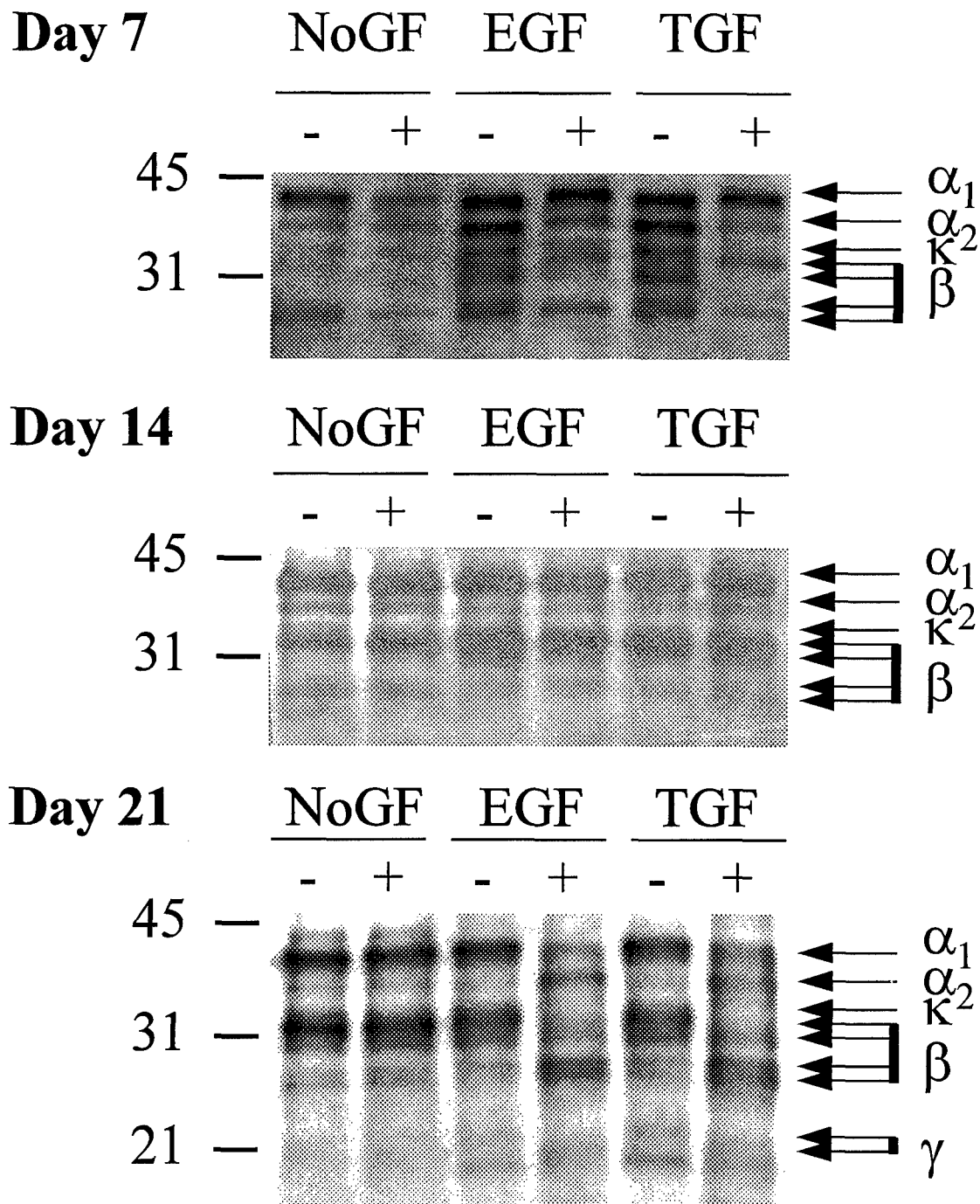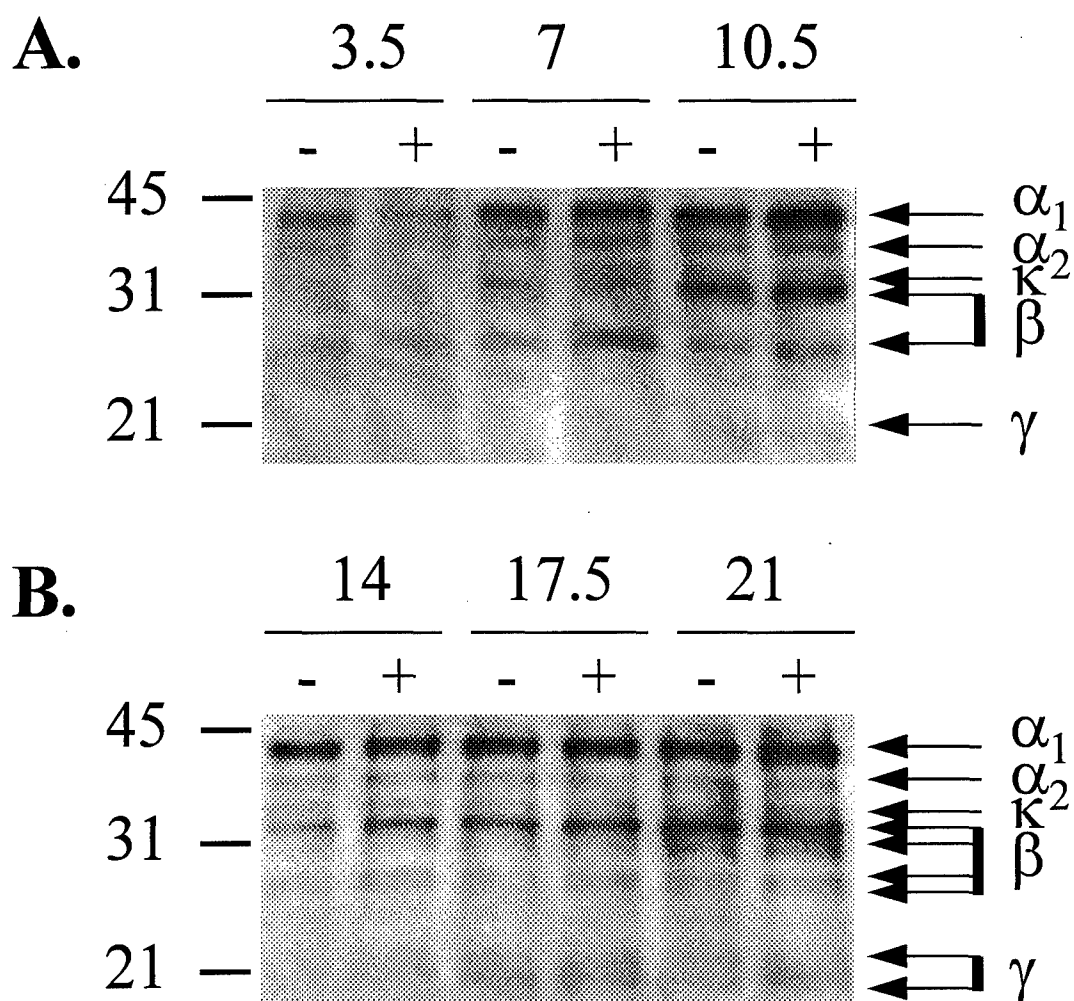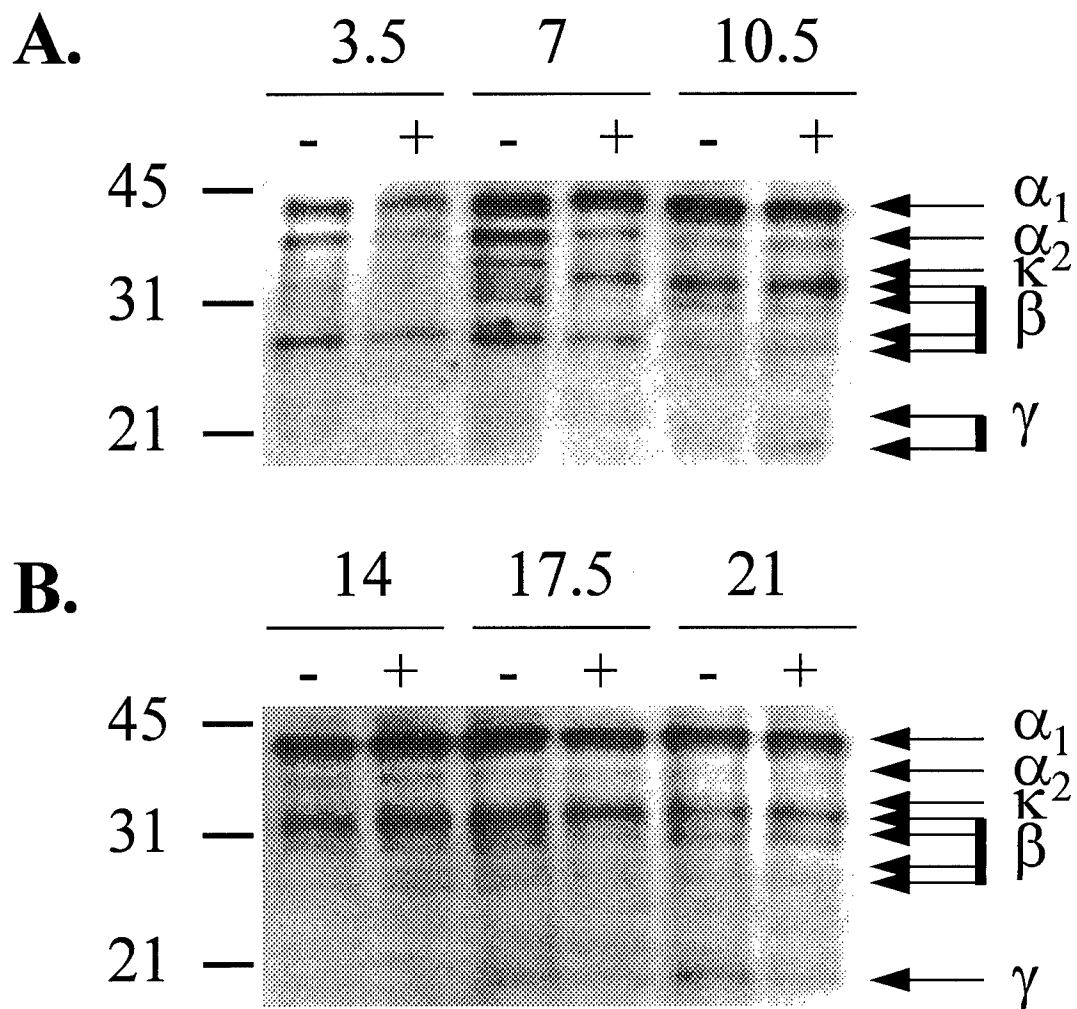
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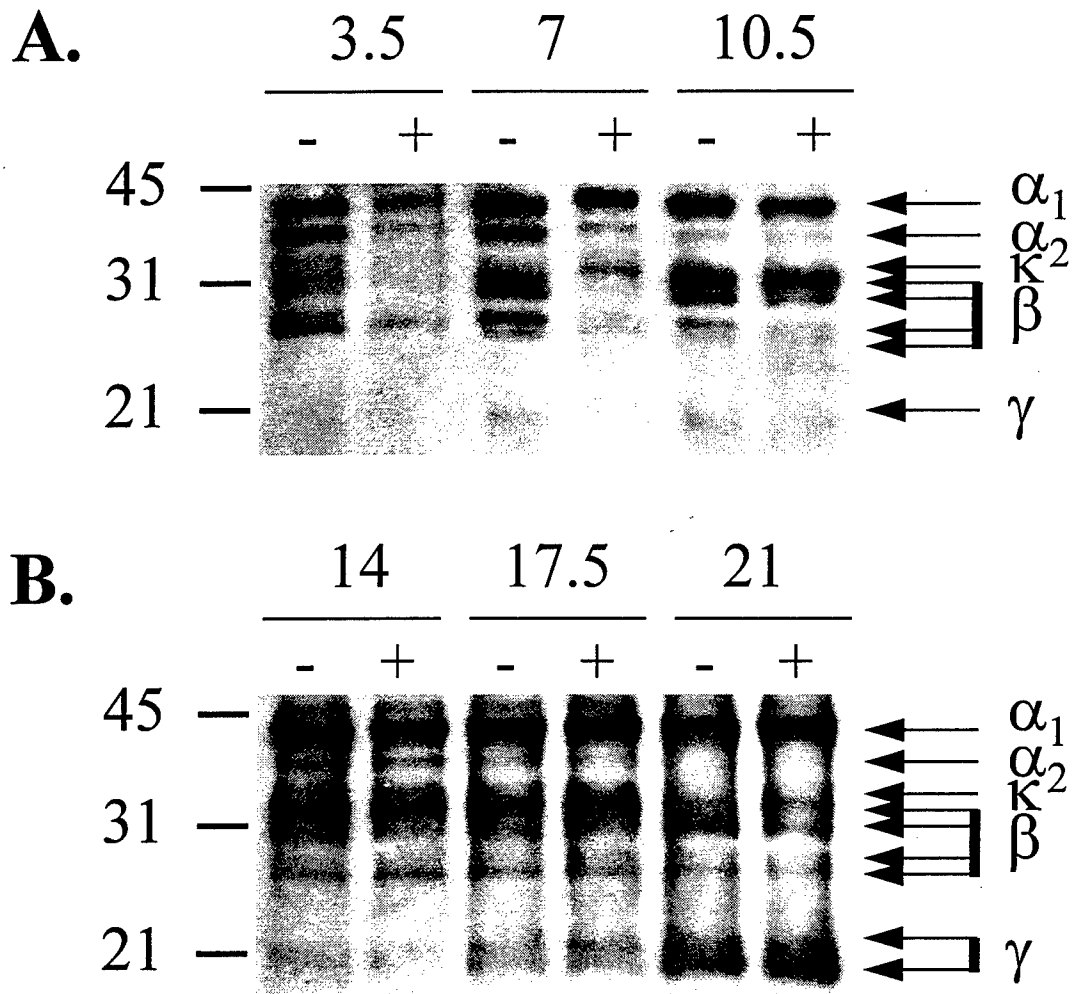
TGFCas1

Figure 10.

A. 10 µg/lane

B. 25 µl/lane

Figure 11.

A. 10 µg/lane
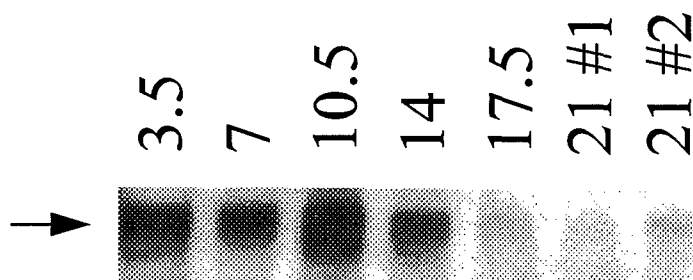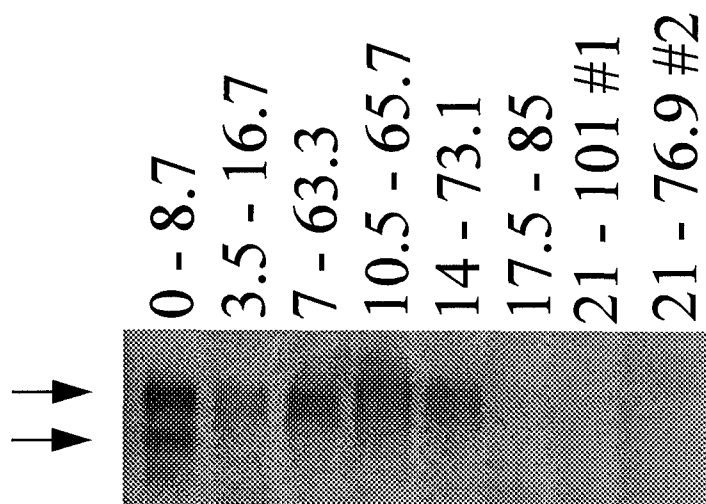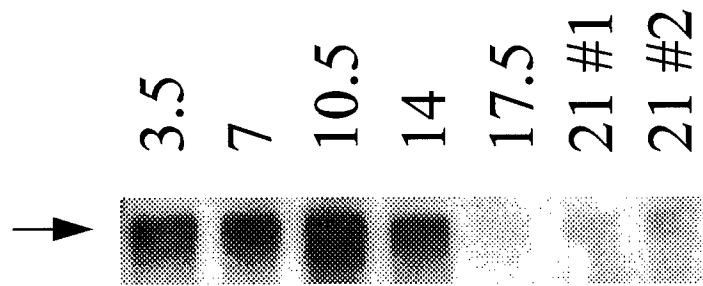
B. 25 µl/lane

28EGFR2

Figure 12.

25 µl/lane

28EGFR3

FIGURE 13.

Figure 14.

A.

V   P   L5 L10 L15 L21 I7

B.

V   P   L5   L10 L15 L21 I7

C.

V   P   L6 L21 I3   I7
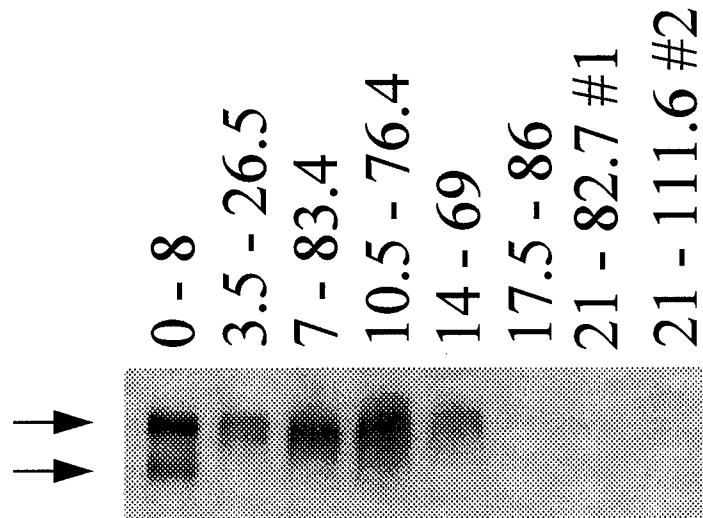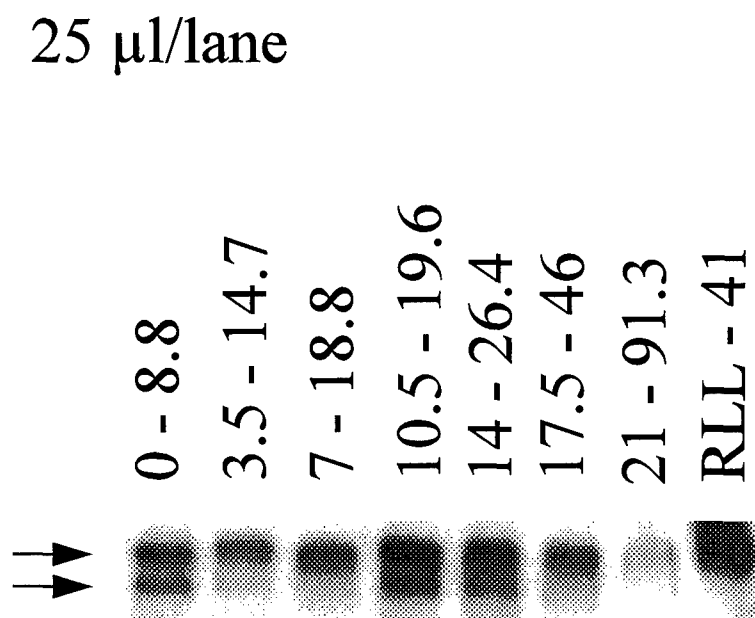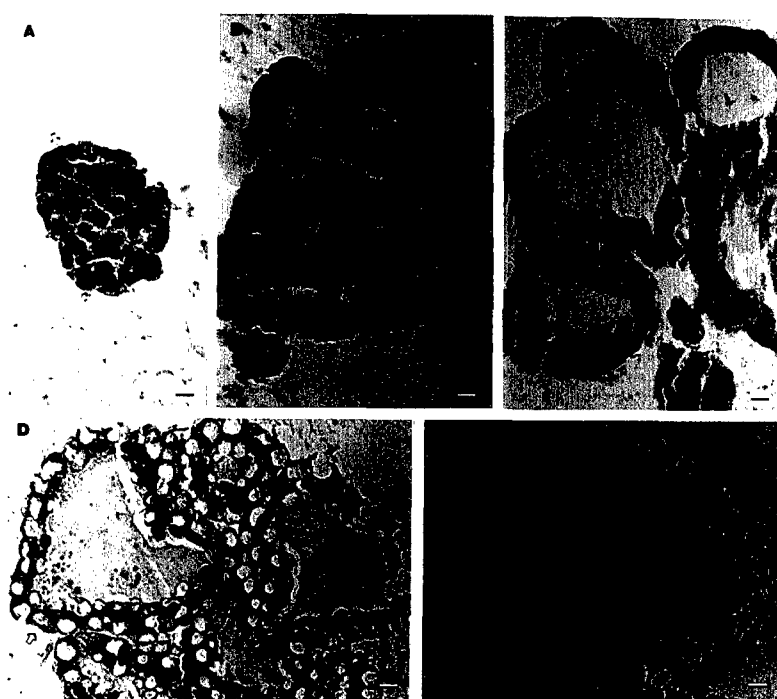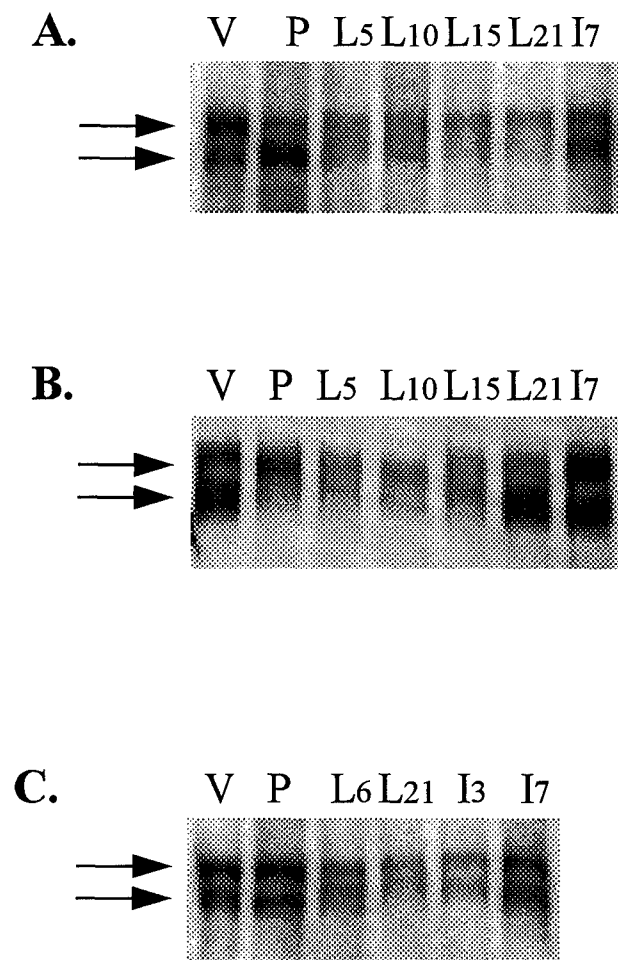
FIGURE 15.

Figure 16.

ErbBMEOE.ppt
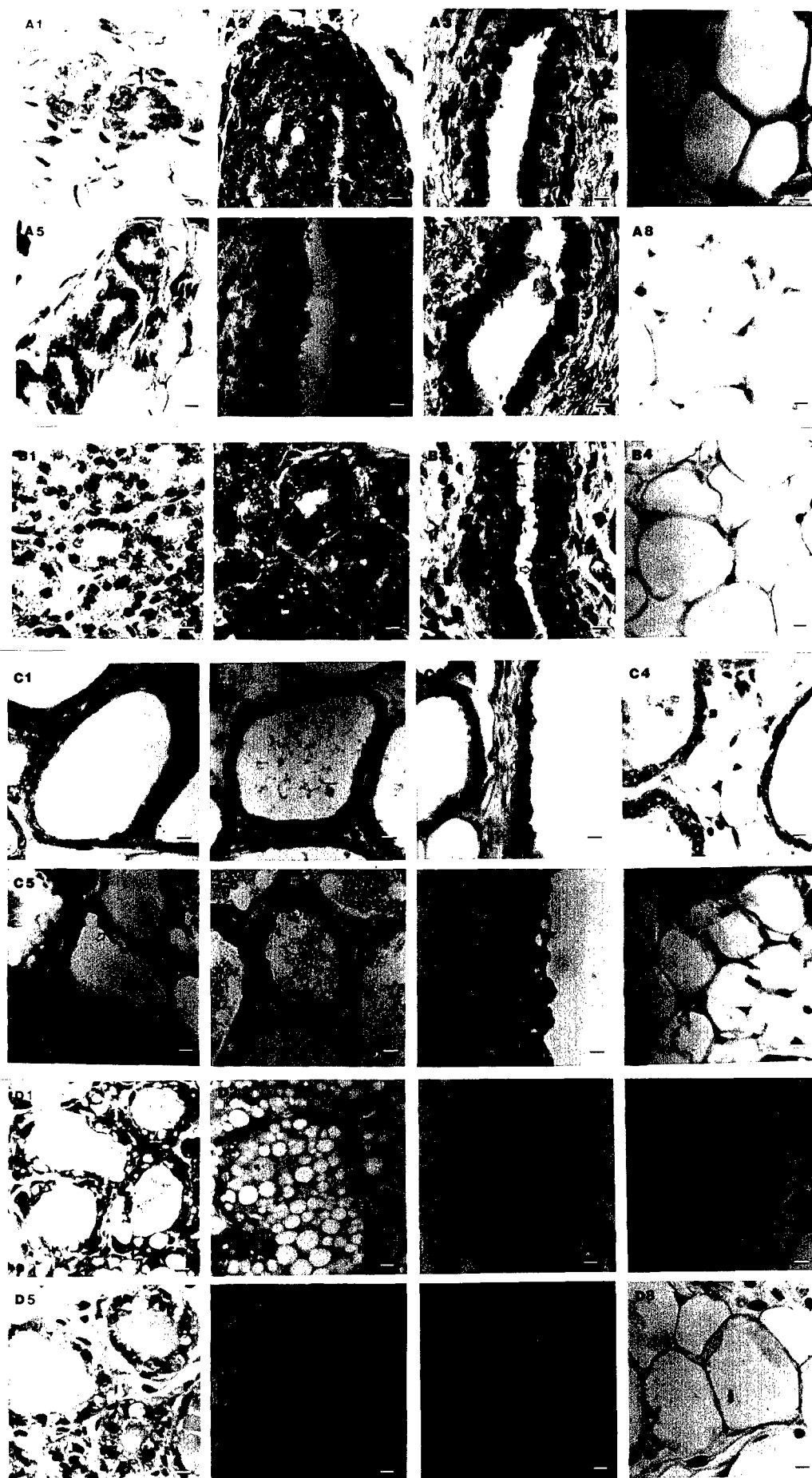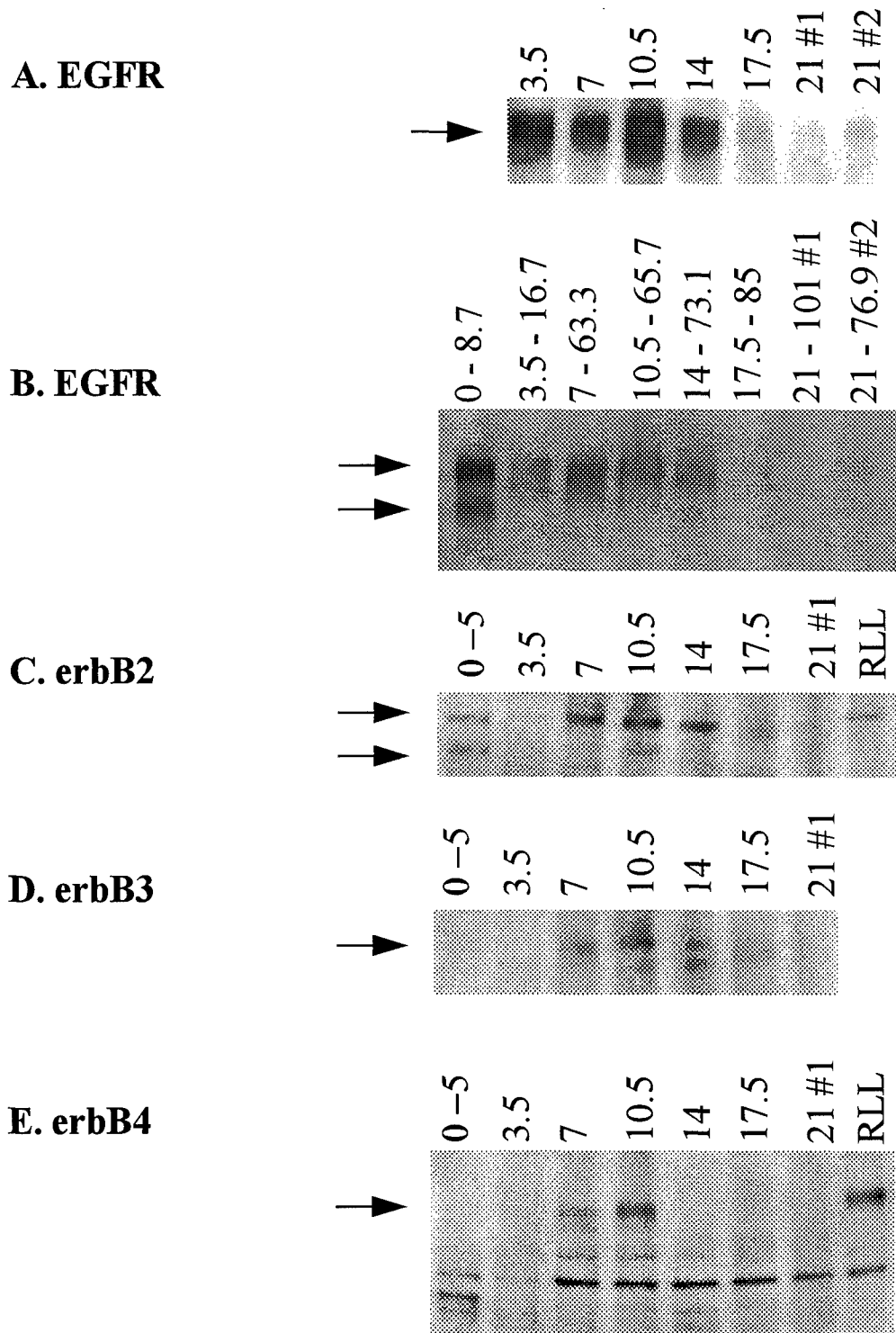
Figure 17.

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A. EGFR

UBI α-EGFR   UBI α-EGFR   Sheep IgG

B. ErbB2

SC-284   SC284+ SC284P Competition   SC-284   SC284+ SC284P Competition

C. ErbB3

SC-285   SC285+ SC285P Competition   SC-285   SC285+ SC285P Competition

D. ErbB4

SC-283   SC283+ SC283P Competition   Rabbit IgG
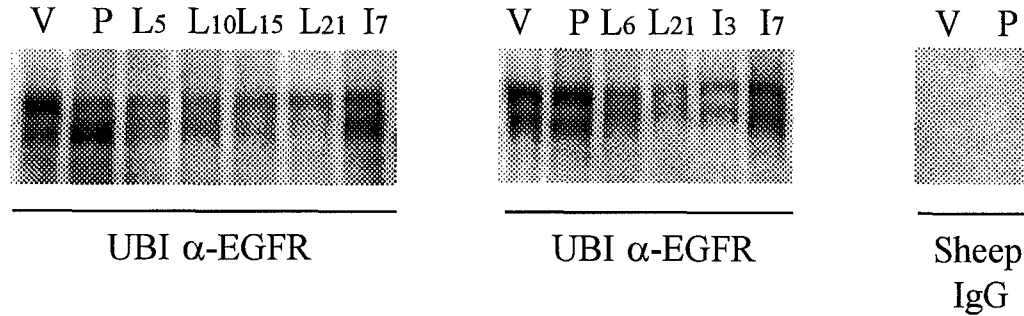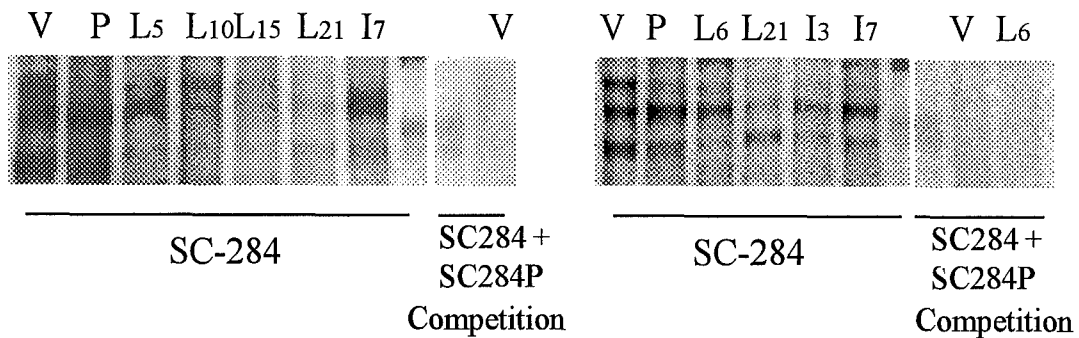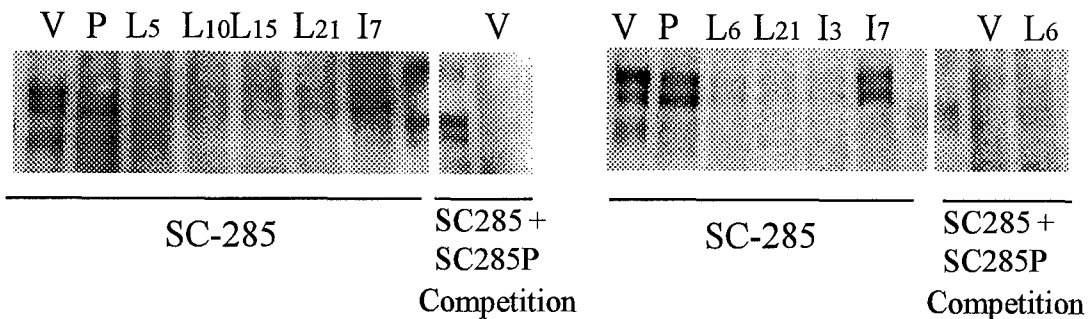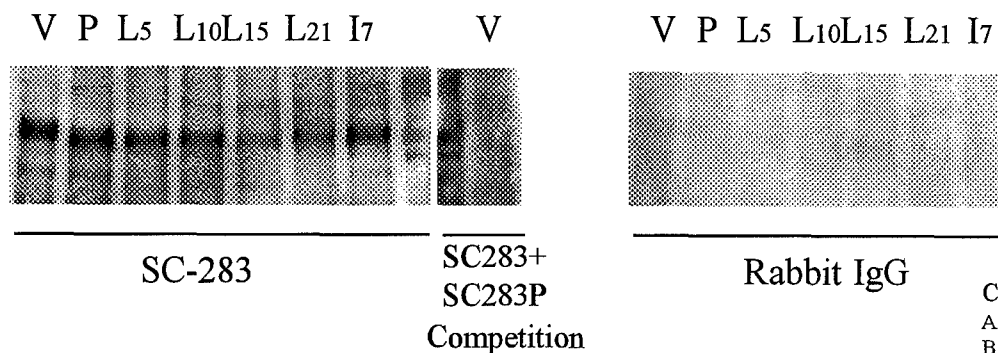
ComErbB.ppt
A - 308 and 316
B - 309 and 314
C - 305 and 315
D - 306 and 307
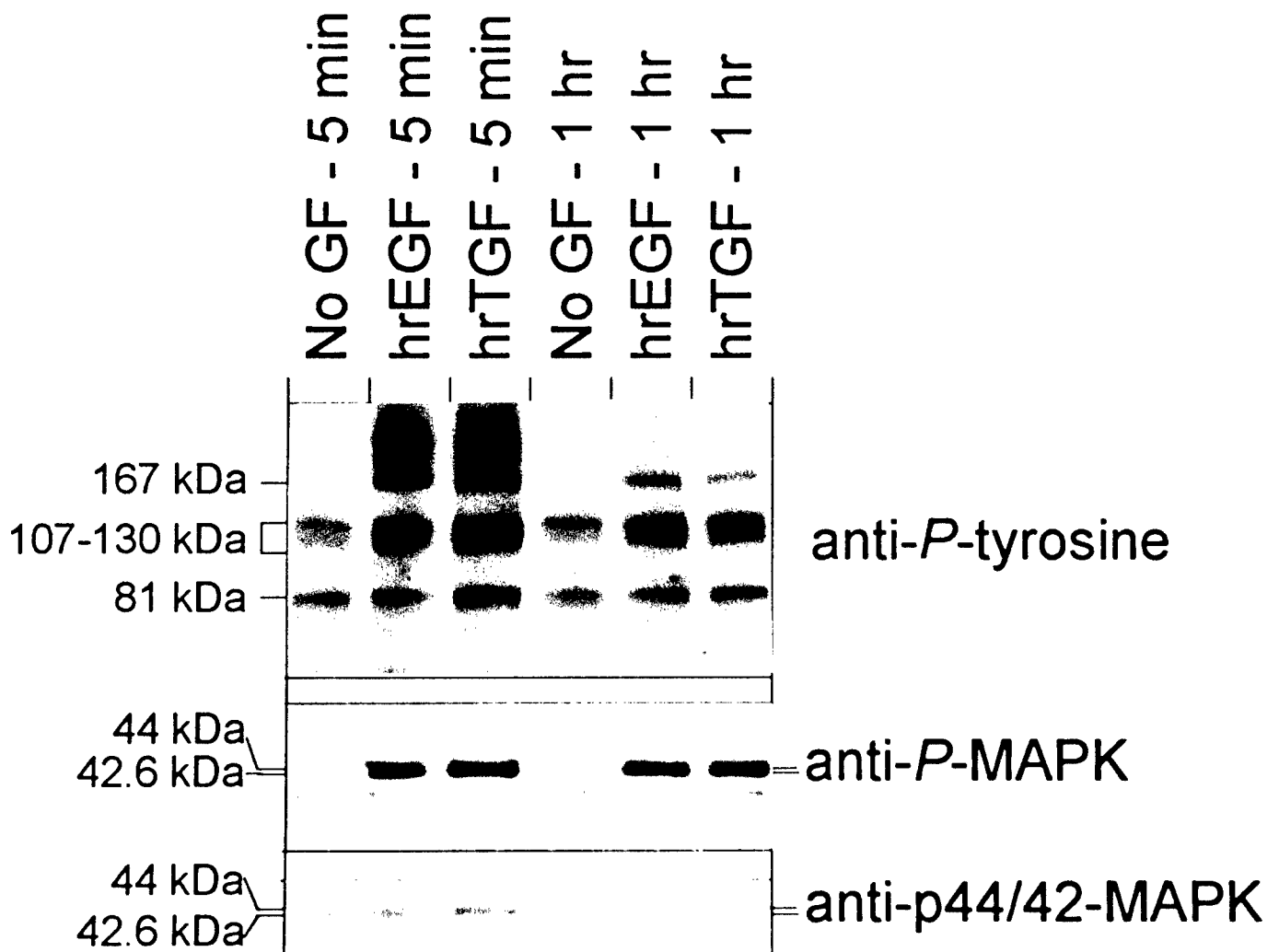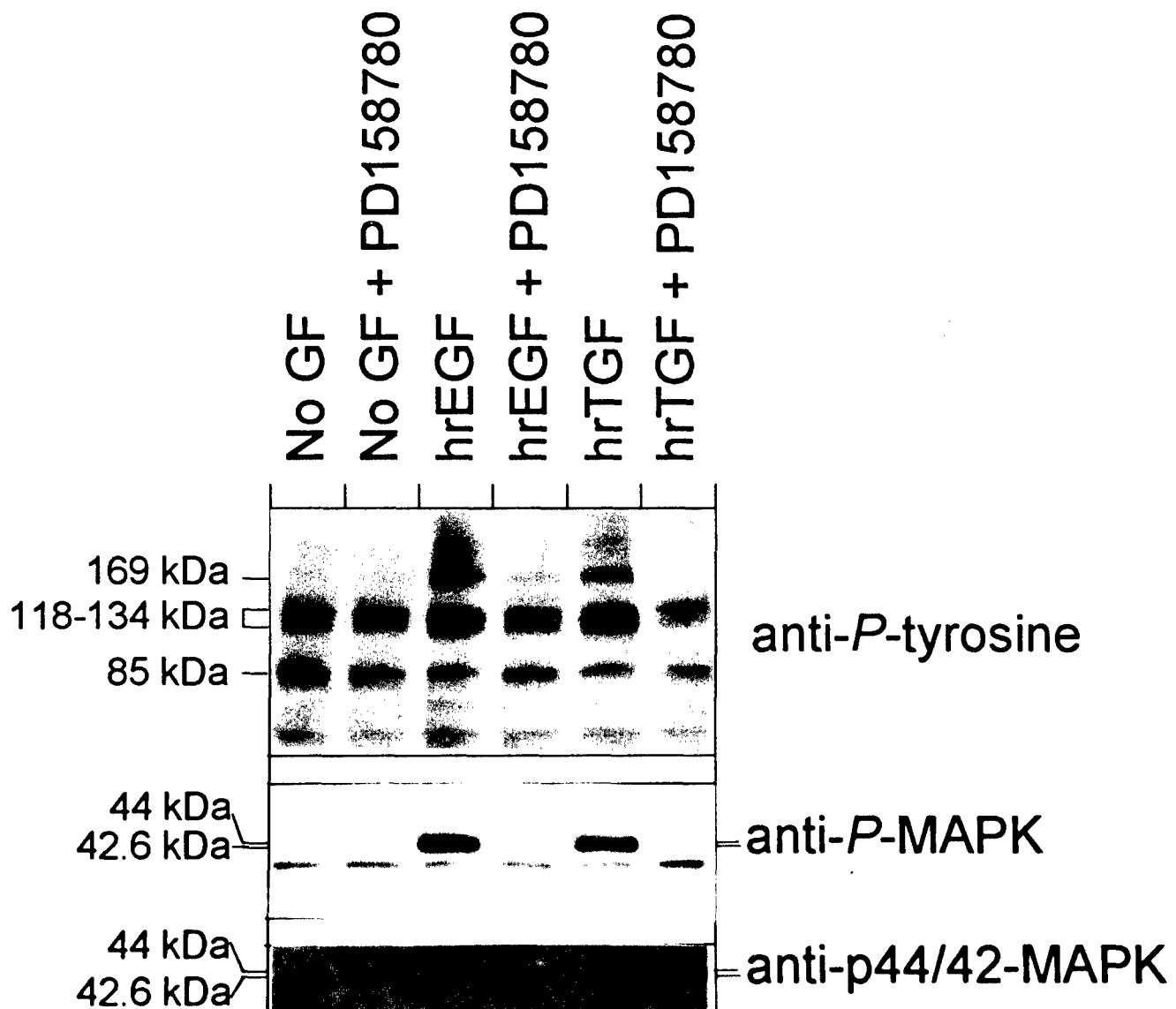
FIGURE 18.
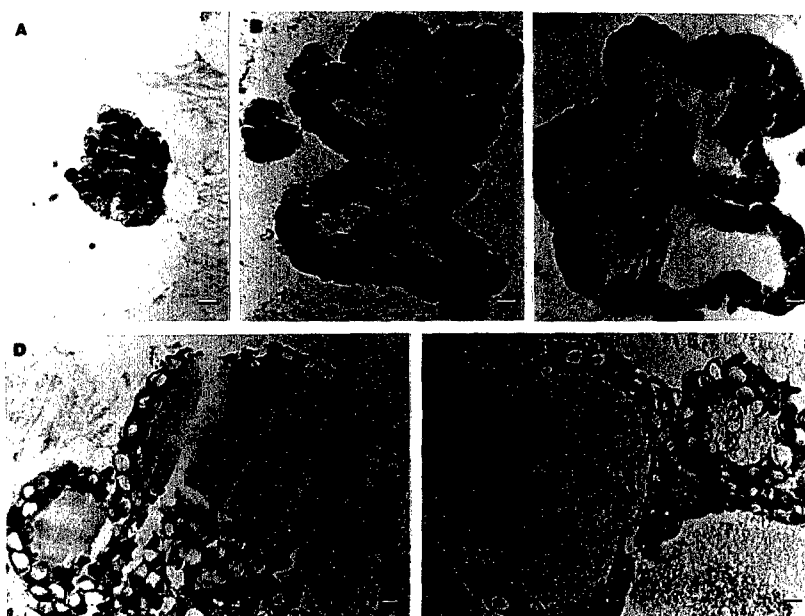
FIGURE 19.
(1 of 2)

FIGURE 20.

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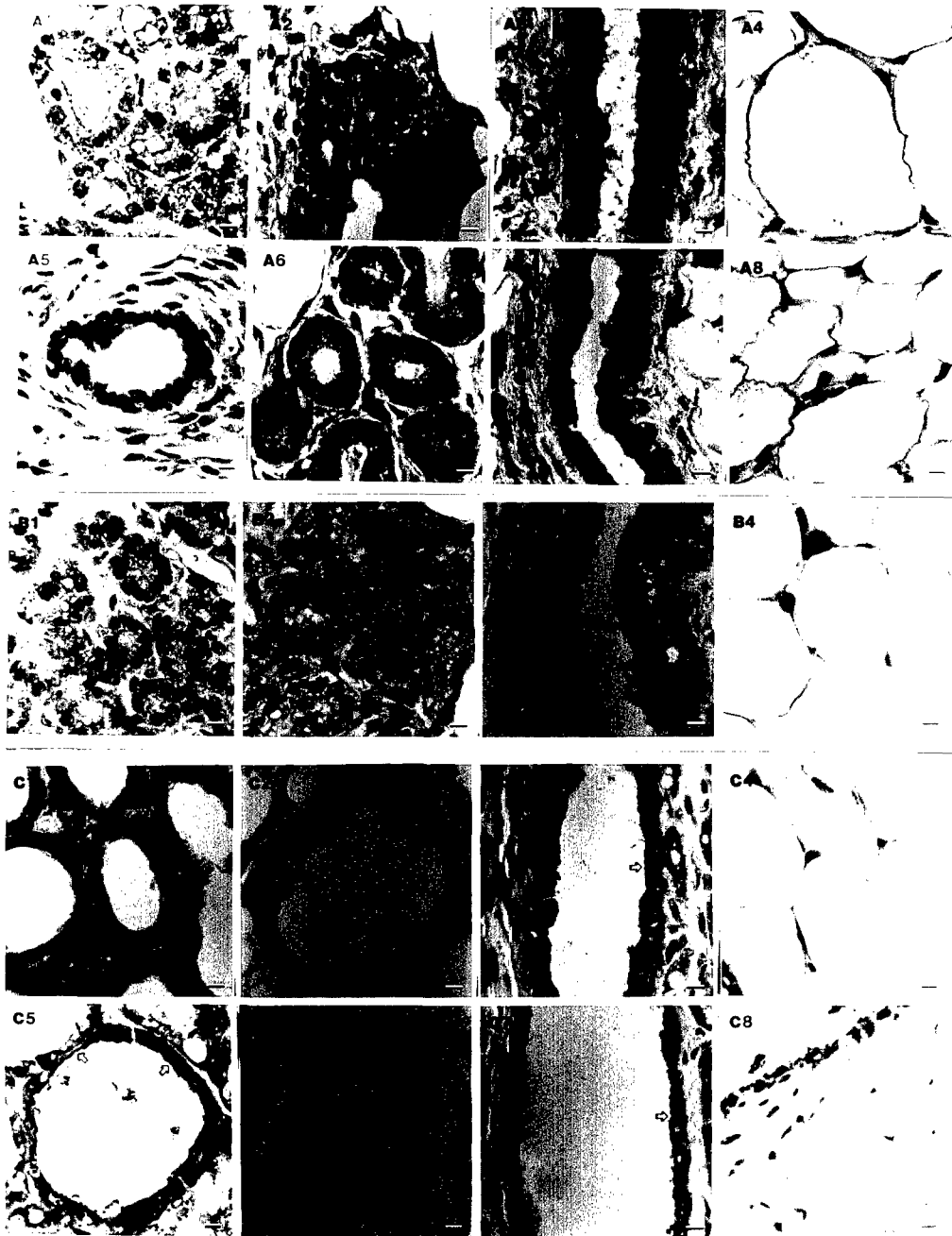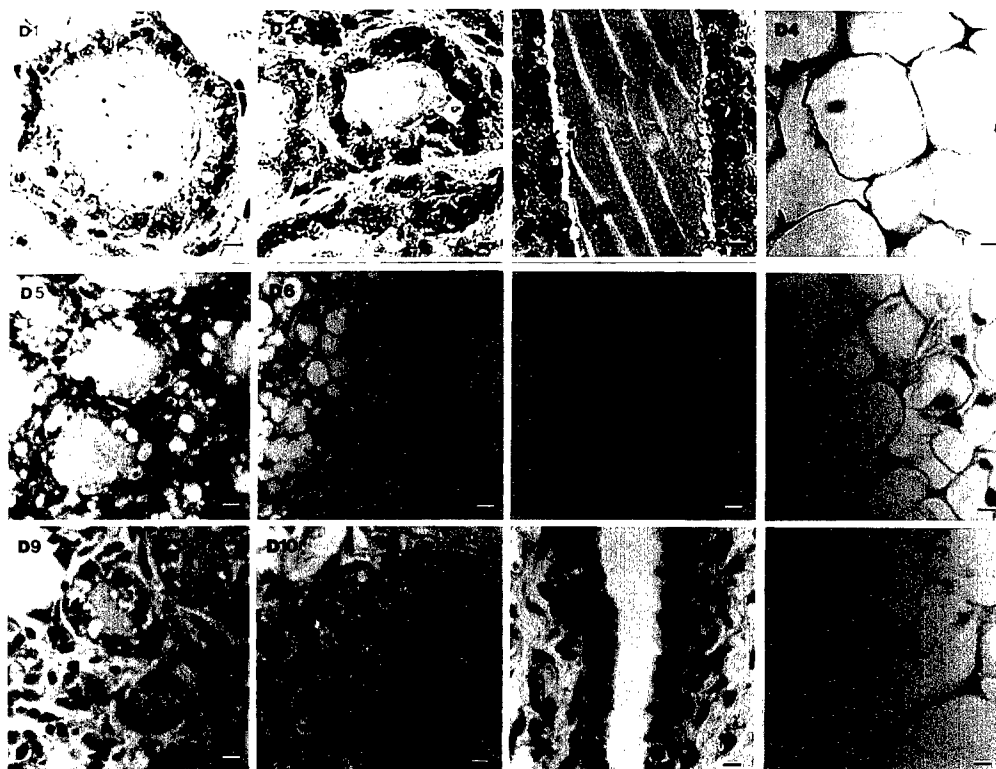
EGFR-Dependent Tyrosine Phosphorylation in Normal Mammary Fibroblasts

FIGURE 21.

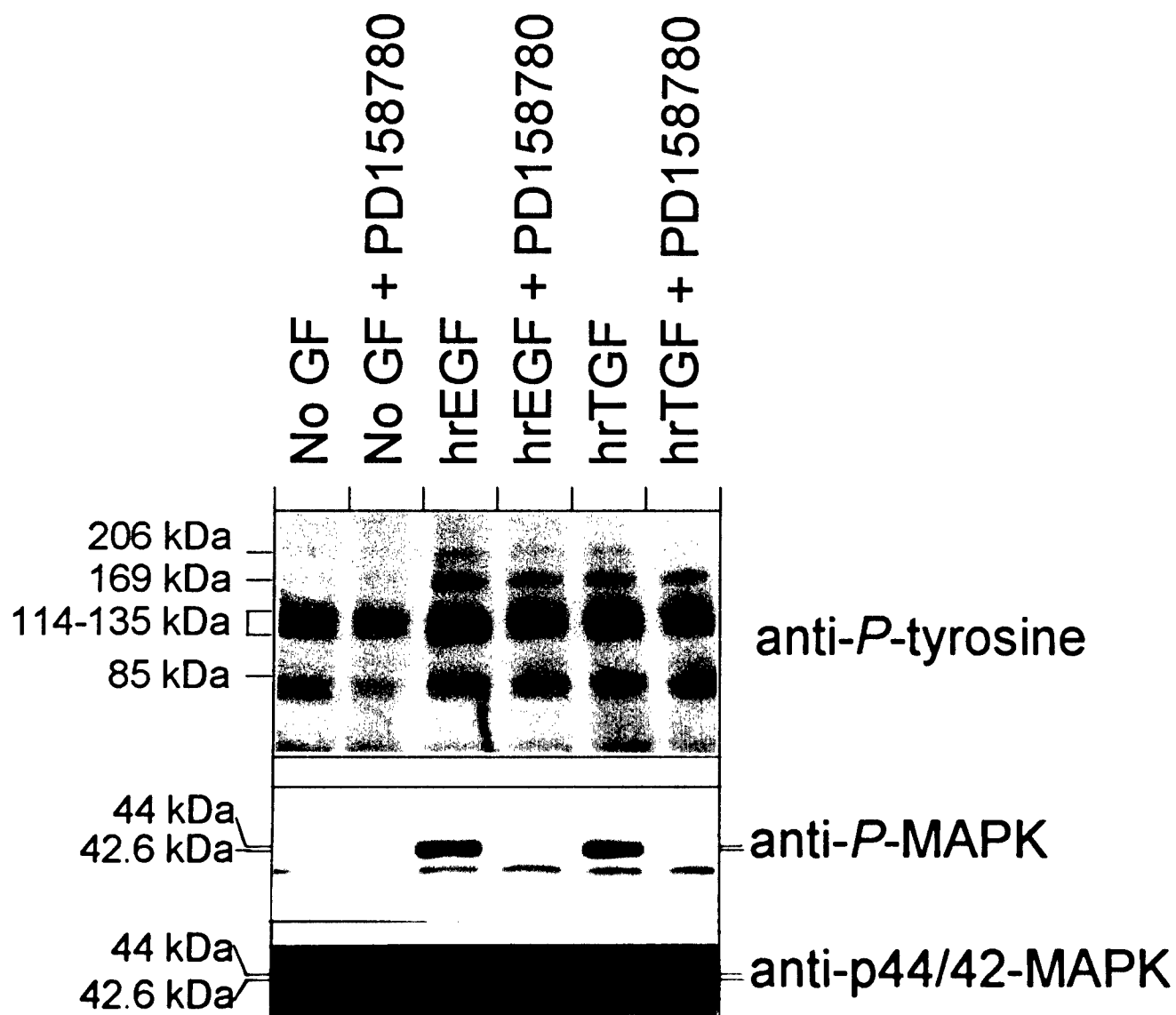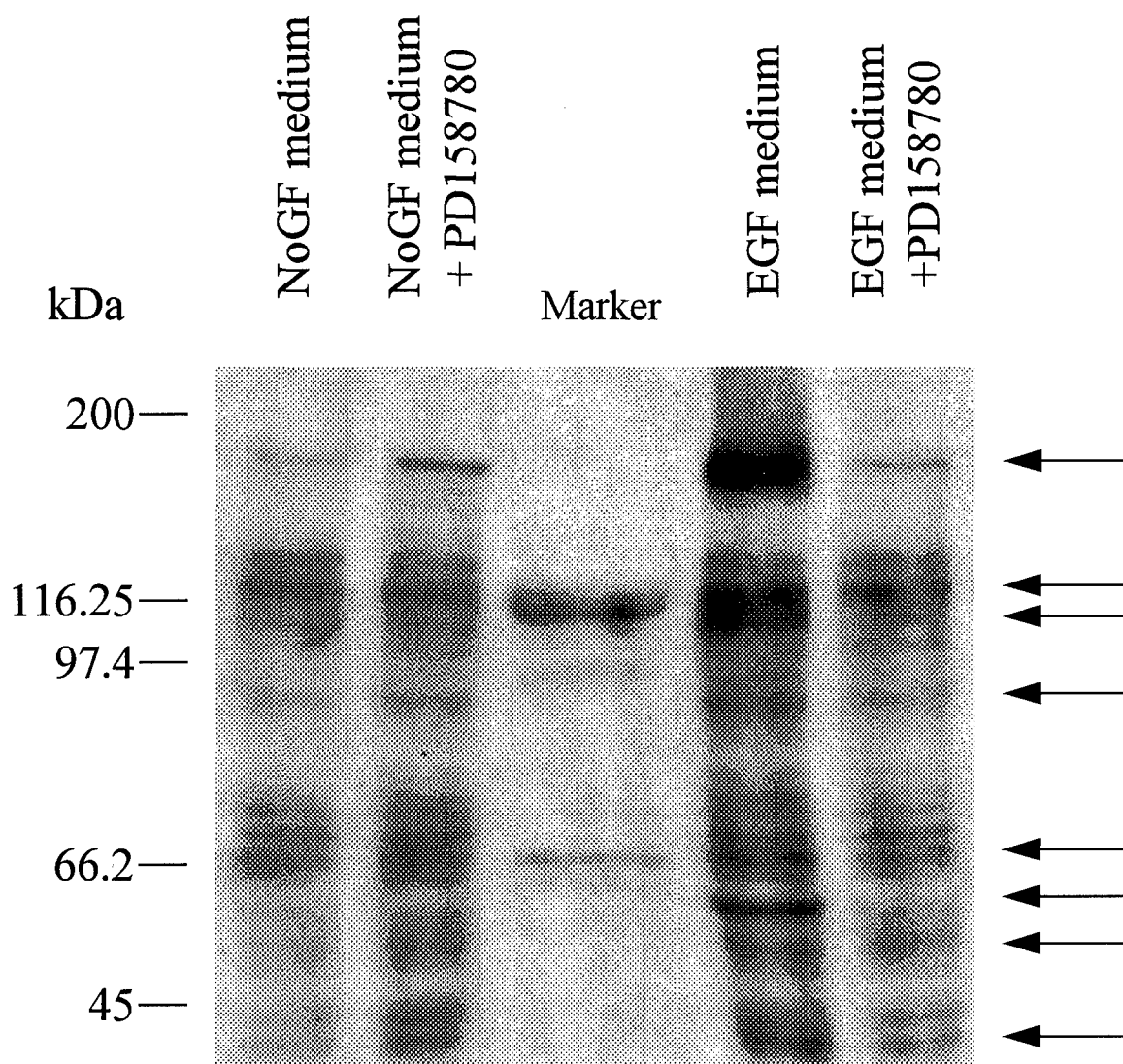# EGFR-Dependent Tyrosine Phosphorylation in Response to a 15 min Treatment

Lanes: No GF | No GF + PD158780 | hrEGF | hrEGF + PD158780 | hrTGF | hrTGF + PD158780

169 kDa
118-134 kDa
85 kDa
— anti-*P*-tyrosine

44 kDa
42.6 kDa
— anti-*P*-MAPK

44 kDa
42.6 kDa
— anti-p44/42-MAPK

FIGURE 22.

EGFR-Dependent Tyrosine Phosphorylation in Response to a 2 hr Treatment

Figure 23.

5297LH12.ppt

Table 1. Mammary epithelial organoids cultured in EGF or TGF Medium, but not in NoGF Medium underwent extensive apoptosis when exposed to the EGFR Inhibitor from Day 17.5-19.5 of culture development.

Different Treatment Groups	Total # of Apoptotic Cells Counted	Total # of Cells Counted	% Apoptotic Cells per Colony n = 100 colonies
NoGF Medium			
+ No PD15870	105	8005	1.64 +/- 0.26
+ 0.5 μM PD158780	172	11356	2.24 +/- 0.27
EGF Medium *			
+ No PD15870	17	11169	0.14 +/- 0.06
+ 0.5 μM PD158780	**4739**	12102	**40.64 +/- 3.54**
TGF Medium *			
+ No PD15870	770	10285	9.15 +/- 1.61
+ 0.5 μM PD158780	**8734**	11606	**76.58 +/- 2.18**

* $p < 0.05$ as determined using a one way ANOVA and the Student-Newman-Kuels test for pairwise multiple comparisons

21 st Meeting of the International Association for Breast Cancer Research

ABSTRACT FORM

TITLE	Role of Epidermal Growth Factor Receptors and their Ligands in the Development of Normal Mammary Epithelial Cells and Mammary Fibroblasts.
AUTHORS ADDRESS	K. Darcy,* A. Wohlhueter, D. Zangani, S. Shoemaker, W. Shea, P.-P. Lee, and M. Ip. Roswell Park Cancer Institute, Buffalo, NY 14263 USA

Epidermal growth factor (EGF) and transforming growth factor-α (TGF-α) exert their effects by binding to a membrane-associated EGF receptor (EGFR) and subsequently activating the tyrosine kinase domain of this receptor. Although their mechanisms of action are not completely understood, these ligands and their receptor are physiological regulators of normal mammary gland development, and are often overexpressed in estrogen receptor negative breast cancers. Initially, we examined the biological effects of EGF and TGF-α on normal rat mammary epithelial cell (MEC) development in primary culture, and then used PD158780, a potent and selective inhibitor of the tyrosine kinase domain of the EGFR, to demonstrate that EGFR signaling is required, at least in part, for normal MEC proliferation, functional differentiation (as assessed by casein accumulation), colony survival, branching morphogenesis, as well as secretion of certain matrix-degrading metalloproteinases. In addition, normal rat mammary fibroblast cells (MFC) were used to examine rapid signal transduction events including tyrosine phosphorylation protein profiles and MAP kinase (MAPK) phosphorylation in response to EGFR activation by EGF or TGF-α. PD158780 was then employed to determine which of the tyrosine phosphorylation events induced in the MFC by EGF or TGF-α were EGFR-dependent. We will continue to examine the role that EGFR plays in the development of normal MEC cultured alone as well as MEC co-cultured with the EGFR-responsive MFC, and then examine their role during rat mammary tumor progression and metastasis. Ultimately, we hope that this data will help to identify new therapeutic targets and develop effective therapies to treat patients with estrogen receptor negative breast cancer. Supported by DAMD17-94-J-4159 and NIH CA 64870.

KEY WORDS	mammary, epithelial, fibroblasts, EGF, TGF-α, EGF receptor, tyrosine phosphorylation

SPECIALTY
(check one or two boxes)

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☐ Psychology
XX Therapeutics
☐ Diagnostic procedures
☐ Nutrition
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<TITLE> Differential effects of epidermal growth factor receptor [EGFR] inhibition in mammary epithelial cells [MEC].
<LAST>Darcy
<INITIAL>K.M.
<LAST>Wohlhueter
<INITIAL>A.
<LAST>Shoemaker
<INITIAL>S.
<LAST>Lee
<INITIAL>P.P.
<LAST>Varela
<INITIAL>L.
<LAST>Ip
<INITIAL>M.M.
<AFFIL>Roswell Park Cancer Institute   Buffalo, NY 14263

<ABSTRACT>EGFR activation regulates various biological effects in normal mammary and breast cancer cells, however, the factors that regulate the type, magnitude and duration of these EGFR-dependent events are poorly understood. To evaluate the biological consequence of EGFR-inhibition on the development of MEC in primary culture, a selective EGFR tyrosine kinase inhibitor, PD158780, was used. Exposure of MEC to 0.5 µM PD158780 from days 0-3.5, 3.5-7, or 0-21 of culture inhibited MEC growth and casein accumulation. Short term treatment of MEC with PD158780 from days 7-10.5, 10.5-14 or 14-17.5 did not consistently affect MEC growth, functional differentiation or survival. In certain studies, however, PD158780 exposure from days 17.5-21 altered casein isoform expression and induced extensive apoptosis. Taken together, these data demonstrate that EGFR activation was required, in part, for the growth, functional differentiation and survival of MEC. They also suggest that EGFR function and/or expression changes during MEC development. It is hoped that by clarifying the changing function of EGFR and its ligands in mammary cells that we may better understand how to treat breast cancer. Support: DAMD17-94-J-4159 and CA33240.

<SPONSOR>3326
<CATEGORY>CG3-03
<TITLE>Stromal-epithelial interactions regulate the *in vitro* development of normal mammary epithelial cells [MEC].
<LAST>Darcy
<INITIAL>K.M.
<LAST>Zangani
<INITIAL>D.
<LAST>Ip
<INITIAL>M.M.
<AFFIL>Roswell Park Cancer Institute   Buffalo, NY 14263
<ABSTRACT>A transwell co-culture model was developed to examine stromal-epithelial interactions that regulate MEC and breast cancer development. Presumptive mesenchymal stem cells [MSC] and MEC were isolated from mammary glands of pubescent female rats.   MSC cultured within a reconstituted basement membrane [RBM] in adipogenic medium differentiated into mature adipocytes [MAC]. In contrast, MSC repeatedly passaged on plastic in stromal medium differentiated into classical fibroblasts.   Interestingly, MSC cultured on a RBM in stromal medium underwent endothelial-like vessel formation reminiscent of embryonic vasculogenesis.

Initial co-culture experiments examined the interactions between MEC and MAC. MEC cultured in transwells without MAC underwent extensive proliferation, branching end bud as well as alveolar morphogenesis, and functional differentiation. Inclusion of MAC in the lower transwell inhibited MEC growth, enhanced casein and lipid accumulation, and preferentially supported alveolar morphogenesis. Future studies will examine the nature of the growth inhibitory as well as the differentiation promoting activities observed in these studies, and then the other types of stromal-epithelial interactions operative in the mammary gland. By studying the factors regulating mesenchymal cell differentiation, and stomal-epithelial interactions we hope to learn how to inhibit breast cancer development. Supported by CA64870 and DAMD17-94-J-4159.

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2. Darcy, K.M., Wohlhueter, A., Shoemaker, S., Lee, P.-P., Varela, L.M., and Ip, M.M., (1997) Differential effects of epidermal growth factor receptor (EGFR) inhibition in mammary epithelial cells (MEC). Proc. Am. Cancer Res. 38: (Abstract)

3. Darcy, K.M., Zangani, D., and Ip, M.M. (1997) Stromal-epithelial interactions regulate the in vitro development on normal mammary epithelial cells [MEC]. Proc. Am. Cancer Res. 38: (Abstract)

4. Darcy, K.M., Wohlhueter, A., Vaughan, M., Russell, J.A., Shoemaker, S., Zangani, D., Huang, R., Varela, L.M., Horn, E., Masso-Welch, P., and Ip, M.M. (manuscript in preparation) Selective changes in EGFR expression and function during the proliferation, differentiation, and apoptosis of normal mammary epithelial cells.

*Kathleen M. Darcy was the sole recipient of payment for these efforts.

DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
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FORT DETRICK, MARYLAND 21702-5012

REPLY TO
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